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(54) Title: **METHOD FOR INTRODUCING AND EXPRESSING GENES IN ANIMAL CELLS, AND BACTERIAL BLEBS FOR USE IN SAME**

(57) Abstract: **A method for introducing and expressing genes in animal cells, in which the animal cells are transfected with bacterial blebs containing a eukaryotic expression cassette encoding the gene. The gene may encode a vaccine antigen, a gene therapeutic agent, an immunoregulatory agent, an anti-sense RNA or a catalytic RNA.**

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METHOD FOR INTRODUCING AND EXPRESSING GENES IN ANIMAL CELLS, AND BACTERIAL BLEBS FOR USE IN SAME

CROSS-REFERENCE TO RELATED APPLICATION

The priority of United States Provisional Patent Application No. 60/206,994 filed May 24, 2000 in the names of David M. Hone and Robert J. Powell for "Method for Inducing Nucleic Acids Into Cells Using Bacterial Blebs as Vectors" is hereby claimed.

DESCRIPTION

FIELD OF THE INVENTION

The present invention relates to a method for introducing endogenous or foreign genes into animal cells using bacterial blebs as vectors. The method allows for the delivery of eukaryotic expression cassettes encoding the endogenous or foreign genes into animal cells or animal tissue, and is useful for expressing, e.g., vaccine antigens, gene therapeutic agents, immunoregulatory agents, antisense RNAs, and catalytic RNAs, in animal cells or animal tissue.

BACKGROUND OF THE INVENTION

I. Live Bacterial Vector Vaccines

The advent of recombinant DNA technology has greatly accelerated the development of vaccines to control epidemic, endemic, and pandemic infectious diseases

(Woodrow et al, New Generation Vaccines. The Molecular Approach, Eds., Marcel Dekker, Inc., New York, NY (1989); Cryz, Vaccines and Immunotherapy, Ed., Pergamon Press, New York, NY (1991); and Levine et al, Ped. Ann., 22:719-725 (1993)). In particular, this technology has enabled the growth of a new class of vaccines called bacterial vector vaccines (Curtiss, In: New Generation Vaccines: The Molecular Approach, Ed., Marcel Dekker, Inc., New York, NY, pages 161-188 and 269-288 (1989); and Mims et al, In: Medical Microbiology, Eds., Mosby-Year Book Europe Ltd., London (1993)). These vaccines can enter the host orally, intranasally or parenterally. Upon gaining access to the host, the bacterial vector vaccines express an engineered prokaryotic expression cassette contained therein that encodes a foreign antigen(s). Foreign antigens can be any protein (or part of a protein) or combination thereof from a bacterial, viral, or parasitic pathogen that has vaccine properties (New Generation Vaccines: The Molecular Approach, supra; Vaccines and Immunotherapy, supra; Hilleman, Dev. Biol. Stand., 82:3-20 (1994); Formal et al, Infect. Immun., 34:746-751 (1981); Gonzalez et al, J. Infect. Dis., 169:927-931 (1994); Stevenson et al, FEMS Lett., 28:317- 320 (1985); Aggarwal et al, J. Exp. Med., 172:1083-1090 (1990); Hone et al, Microbial. Path., 5:407-418 (1988); Flynn et al, Mol. Microbiol., 4:2111-2118 (1990); Walker et al, Infect. Immun., 60:4260-4268 (1992); Cardenas et al, Vacc., 11:126-135 (1993); Curtiss et al, Dev. Biol. Stand., 82:23-33 (1994); Simonet et al, Infect. Immun., 62:863-867 (1994); Charbit et al, Vacc., 1-1:1221-1228 (1993); Turner et al, Infect. Immun., 61:5374-5380 (1993); Schodel et al, Infect. Immun., 62:1669-1676 (1994); Schodel et al, J. Immunol., 145:4317-4321 (1990); Stabel et al, Infect. Immun., 59:2941-2947 (1991); Brown, J. Infect. Dis., 155:86-92 (1987); Doggett et al, Infect. Immun., 61:1859-1866 (1993); Brett et al, Immunol., 80:306-312 (1993); Yang et al, J. Immunol., 145:2281-2285 (1990); Gao et al, Infect.

Immun., 60:3780-3789 (1992); and Chatfield et al, Bio/Technology, 10:888-892 (1992)). Delivery of the foreign antigen to the host tissue using bacterial vector vaccines results in host immune responses against the foreign antigen, which provide protection against the pathogen from which the foreign antigen originates (Mims, The Pathogenesis of Infectious Disease, Academic Press, London (1987); and New Generation Vaccines: The Molecular Approach, supra).

Of the bacterial vector vaccines, live oral Salmonella vector vaccines have been studied most extensively. There are numerous examples showing that Salmonella vectors are capable of eliciting humoral and cellular immunity against bacterial, viral and parasitic antigens (Formal et al, Infect. Immun., 34:746-751 (1981); Gonzalez et al, supra; Stevenson et al, supra; Aggarwal et al, supra; Hone et al, supra; Flynn et al, supra; Walker et al, supra; Cardenas et al, supra; Curtiss et al, supra; Simonet et al, supra; Charbit et al, supra; Turner et al, supra; Schodel et al, supra, Schodel et al (1990), supra; Stabel et al, supra; Brown, supra; Doggett et al, supra; Brett et al, supra; Yang et al, supra; Gao et al, supra; and Chatfield et al, supra). These humoral responses occur in the mucosal (Stevenson et al, supra; Cardenas et al, supra; Walker et al, supra; and Simonet et al, supra), and systemic compartments (Gonzalez et al, supra; Stevenson et al, supra; Aggarwal et al, supra; Hone et al, supra; Flynn et al, supra; Walker et al, supra; Cardenas et al, supra; Curtiss et al, supra; Simonet et al, supra; Charbit et al, supra; Turner et al, supra; Schodel et al, supra; Schodel et al (1990), supra; Stabel et al, supra; Brown, supra; Doggett et al, supra; Brett et al, supra; Yang et al, supra; Gao et al, supra; and Chatfield et al, supra). Live oral Salmonella vector vaccines also elicit T cell responses against foreign antigens (Wick et al, Infect. Immun., 62:4542-4548 (1994)). These include antigen-specific cytotoxic

CD8⁺ T cell responses (Gonzalez et al, supra; Aggarwal et al, supra; Flynn et al, supra; Turner et al, supra; and Gao et al, supra).

Ideally, bacterial vector vaccines are genetically defined, attenuated and well-tolerated by the recipient animal or human, and retain immunogenicity (Hone et al, *Vacc.*, 9:810-816 (1991); Tacket et al, *Infect. Immun.*, 60:536-541 (1992); Hone et al, *J. Clin. Invest.*, 90:412-420 (1992); Chatfield et al, *Vacc.*, 10:B-11 (1992); Tacket et al, *Vacc.*, 10:443-446 (1992); and Mims, supra). Recently, the number of potential bacterial vector vaccines for the delivery of prokaryotic expression cassettes has grown. They now include, but are not restricted to, *Yersinia enterocolitica* (van Damme et al, *Gastroenterol.*, 103:520-531 (1992)), *Shigella* spp. (Noriega et al, *Infect. Immun.*, 62:5168-5172 (1994)), *Vibrio cholerae* (Levine et al, In: *Vibrio cholerae, Molecular to Global Perspectives*, Wachsmuth et al, Eds, ASM Press, Washington, D.C., pages 395-414 (1994)), *Mycobacterium* strain BCG (Lagranderie et al, *Vacc.*, 11:1283-1290 (1993); Flynn, *Cell. Molec. Biol.*, 40(Suppl. 1):31-36 (1994)), and *Listeria monocytogenes* (Schafer et al, *J. Immunol.*, 149:53-59 (1992)) vector vaccines.

II. Bacterial Blebs

During the growth of bacteria small membrane vesicles are produced, which are colloquially called blebs (Dorward et al., *J. Bacteriol.*, 171:2499 (1989)). Bacteria that make blebs include but are not limited to *Agrobacterium* spp., *Bacillus* spp., *Borrelia* spp., *Bordetella* spp., *Calymmatobacterium* spp., *Escherichia* spp., *Haemophilus* spp., *Neisseria* spp., *Pseudomonas* spp., *Porphyromonas* spp., *Fusobacterium* spp., *Rhodococcus* spp., *Salmonella* spp., *Serratia* spp., *Shigella* spp.,

and *Yersinia* spp. (Dorward and Garon, *Appl. Environ. Microbiol.*, 56:1960 (1990); Karow et al, *J. Bact.*, 173:741-750 (1991); Karow and Georgopoulos, *J. Bact.*, 174:702-710 (1992); Begg and Donachie, *J. Bact.*, 163:615-622 (1985); Radolf et al, *J. Bact.*, 176:21-31 (1994); Whitmire and Garon, *Infect. Immun.*, 61:1460-1467 (1993); Garcia et al, *Canad. J. Vet. Res.*, 56:318-325 (1992);, and Chandra and Jain, *Ind. J. Med. Res.*, 93:225- 231 (1991). The envelope of bacterial blebs can be composed of outer membrane, inner membrane or a combination of both (Dorward et al, *supra*). The latter are more correctly called mini-cells (Adler et al., *Proc. Natl. Acad. Sci., USA*, 5-7:321-326 (1967); and de Boer et al, *Cell*, 56:641-649 (1989)). Each of these fractions can be isolated by differential centrifugation followed by sucrose or cesium chloride density gradient centrifugation (Dorward et al., *supra*). Some bacterial strains are naturally high producers of blebs (Dorward and Garon, *supra*). However, certain mutations that result in defective cell wall synthesis increase the frequency of bleb formation in strains that are low producers of blebs (Adler et al., *supra*; de Boer et al, *supra*; Karow et al, *supra*; Karow and Georgopoulos, *supra*; and Begg and Donachie, *supra*). Blebs can also be produced artificially by physical disruption methods such as sonication of bacterial cells or passing bacteria through a French pressure cell (Morein et al, *Anal. Biochem.*, 216:47-51 (1994)); or by treatment of bacterial cells with bacteriocidal or bacteriostatic chemicals (Kadurugamuwa et al, *J. Bact.*, 175:5798-5805 (1993); and Dargis et al, *Infect. Immun.*, 60:4024-4031 (1992)).

Blebs can contain bacterial chromosomal and/or plasmid DNA (Dorward and Garon, *supra*). It is not clear how the DNA becomes associated with the blebs. Interestingly, blebs have been shown to transfer plasmid DNA within a bacterial species (Dorward

et al., supra). This phenomenon has been proposed as a alternative method of genetic exchange between bacteria (Dorward et al., supra); Holloway, *Ann. Rev. Microbiol.*, 47:659 (1993); Dreiseikelmann, *Microbiol. Rev.*, 58:293 (1994)).

III. Eukaryotic and Prokaryotic

Expression Cassettes

Normally, an expression cassette is composed of a promoter region, a transcriptional initiation site, a ribosome binding site (RBS), an open reading frame (orf) encoding a protein (or fragment thereof), with or without sites for RNA splicing (only in eukaryotes), a translational stop codon, a transcriptional terminator and post-transcriptional poly-adenosine processing sites (Wormington, *Curr. Opin. Cell Biol.*, 5:950-954 (1993); Reznikoff et al, *Maximizing Gene Expression*, Eds., Butterworths, Stoneham, MA (1986). The promoter region, the RBS, the splicing sites, the transcriptional terminator and post-transcriptional poly-adenosine processing sites are markedly different in eukaryotic expression cassettes than those found in prokaryotic expression cassettes (Wasylyk, In: *Maximizing Gene Expression*, supra, pages 79-99; Reznikoff et al, In: *Maximizing Gene Expression*, supra, pages 1-34; and Lewin, *Genes V*, Oxford University Press, Oxford (1994)). These differences prevent expression of prokaryotic expression cassettes in eukaryotic cells and visa versa (Miller et al, *Mol. Micro.*, 4:881-893 (1990); and Williamson et al, *Appl. Env. Micro.*, 60:771-776 (1994)).

Prokaryotic promoters are not active in eukaryotic cells and eukaryotic promoters are not active in prokaryotic cells (Eick et al, *Trends in Genetics*, 10:292-296 (1994)). The basis for the functional diversity of eukaryotic versus prokaryotic promoters is

mediated by RNA-polymerase, transcription regulatory factors and the DNA structure of the promoter (Eick et al, *supra*).

RNA polymerases are DNA-binding proteins that recognize specific sequences in the DNA of promoter regions. RNA polymerases catalyze the synthesis of RNA molecules by polymerizing nucleoside triphosphates in the specific order that is dictated by the DNA coding sequence (Libby et al, *Mol. Micro.*, 5:999-1004 (1991) Kerppola et al, *FASEB J.*, 5:2833-2842 (1991); Alberts et al, *Molecular Biology of the Cell*, Eds., Garland Publishing Inc., New York, NY (1994); Watson et al, *Molecular Biology of the Gene*, Vol. 1, Eds., The Benjamin/Cummings Publishing Comp. Inc., Menlo Park CA (1987); and Lewin, *supra*). RNA polymerases of prokaryotes typically are composed of two identical α subunits and two similar, but non-identical, β and β' , subunits (Ishihama, *Mol. Micro.*, 6:3283-3288 (1992); Watson et al, *supra*; Alberts et al, *supra*; and Lewin, *supra*). The specificity of prokaryotic RNA polymerases for a given promoter region is mediated by specific σ factors that recognize core sequences encoded by the DNA in the promoter regions (Libby et al, *supra*; and Lewin, *supra*).

In eukaryotic cells, there are three RNA polymerases. Each of the different RNA polymerases contain 10 to 15 different subunits and each performs a different function (Kerppola et al, *supra*; Larson et al, *Biochem. Cell. Biol.*, 69:5-22 (1991); Archambault et al, *Microbiol. Rev.*, 57:703-724 (1993); Watson et al, *supra*; Alberts et al, *supra*; and Lewin, *supra*). In addition, specific DNA-binding factors may be required for the association of eukaryotic RNA polymerases to the DNA in a promoter region (Darnell et al, *Molecular Cell Biology*, Scientific American Books,

Inc., W.H. Freeman and Co., New York, NY (1990); Hori et al, *Curr. Opin. Gen. Devel.*, 4:236-244 (1994); Lewin, *supra*; Watson et al, *supra*; and Alberts et al, *supra*). These binding factors recognize specific sequences in the DNA, and also interact with the eukaryotic RNA polymerases.

There is little similarity between the primary DNA sequence of prokaryotic promoters and the primary DNA sequence of eukaryotic promoters. The DNA structure of prokaryotic promoters is relatively simple, consisting of -10 and -35 regions and proximal regulatory sequences (Darnell et al, *supra*; Lewin, *supra*; Watson et al, *supra*; and Alberts et al, *supra*). Prokaryotic promoters are located upstream of the transcription start site. Eukaryotic promoters, in contrast, are composed of a core unit, which is usually located from 50 bp upstream to 20 bp downstream of the transcriptional start site (Darnell et al, *supra*; Lewin, *supra*; Watson et al, *supra*; and Alberts et al, *supra*), and enhancer sequences that are located from about 100 to 200 bp upstream of the transcriptional start, but also can be located in distal locations (Sonenberg, *Curr. Opin. Gen. Devel.*, 4:310-315 (1994); Geballe et al, *Trends Bio. Sci.*, 1:159-164 (1994); and Lewin, *supra*).

The RBS is the site recognized by the ribosome for binding to the 5' end of messenger RNA (mRNA) molecules. This binding is essential for the translation of mRNA into a protein by the ribosome. In prokaryotes, the RBS in the 5' end of the mRNA molecule is a sequence that is complementary to the 3' end of the small ribosomal RNA molecule (5S rRNA) (Chatterji et al, *Ind. J. Biochem. Biophys.*, 29:128-134 (1992); and Darnell et al, *supra*; Lewin, *supra*; Watson et al, *supra*; and Watson et al, *supra*). The presence of the RBS promotes binding of the ribosome to the 5' end of

the nascent mRNA molecule. Translation is then initiated at the first AUG codon encountered by the scanning ribosome (Darnell et al, supra; Lewin, supra; Watson et al, supra; and Alberts et al, supra). At present, no such recognition pattern has been observed in eukaryotic mRNA-ribosome interactions (Eick et al, supra). In addition, prior to initiation of translation of eukaryotic mRNA, the 5' end of the mRNA molecule is "capped" by addition of methylated guanylate to the first mRNA nucleotide residue (Darnell et al, supra; Lewin, supra; Watson et al, supra; and Alberts et al, supra). It has been proposed that recognition of the translational start site in mRNA by the eukaryotic ribosomes involves recognition of the cap, followed by binding to specific sequences surrounding the initiation codon on the mRNA. It is possible for cap independent translation initiation to occur and/or to place multiple eukaryotic coding sequences within a eukaryotic expression cassette if a cap-independent translation enhancer (CITE) sequence, such as derived from encephalomyocarditis virus (Duke et al., J. Virol., 66:1602-1609 (1992)), is included prior to or between the coding regions (Morgan et al., Nuc. Acids Res., 20:1293-1299 (1992)). However, the initiating AUG codon is not necessarily the first AUG codon encountered by the ribosome (Louis et al, Molec. Biol. Rep., 13:103-115 (1988); and Voorma et al, Molec. Biol. Rep., 19:139-145 (1994); Lewin, supra; Watson et al, supra; and Alberts et al, supra). Thus, RBS binding sequences in eukaryotes are sufficiently divergent from that of prokaryotic RBS such that the two are not interchangeable.

Termination of transcription in prokaryotes is mediated by rho-independent and rho-dependent stem loops (Richardson, Crit. Rev. Biochem. Mol. Biol., 28:1-30 (1993); Platt, Molec. Microbiol., 11:983-990 (1994); and Lewin, supra). In contrast,

terminator loops are not commonly found downstream of eukaryotic expression cassettes, and transcription often extends beyond the end of the open reading frame (Tuite et al, Mol. Biol. Reps., 19:171-181 (1994). However, usually the over-extended mRNA is specifically cleaved by endonucleases, and the 3' end of the mRNA is poly-adenylated by poly-A polymerase (Proudfoot, Cell, 64:671-674 (1991); Wahle, Bioassays, 14:113-118 (1992); Lewin, supra; and Watson et al, supra). Sequences that are recognized by the endonucleases and poly-A polymerase must be included in the 3' end of the mRNA molecule for these post-translation modifications to occur. Polyadenylation of the 3' end of mRNA molecules is thought to be a necessary step prior to transport of mRNA to the cytoplasm of eukaryotic cells and translation into proteins (Sachs et al, J. Biol. Chem., 268:22955-22958 (1993); and Sachs, Cell, 74:413-421 (1993)). A eukaryotic expression cassette does not need to code for a functional gene product, i.e., it may also code for a partial gene product which acts as an inhibitor of a eukaryotic enzyme (Warne et al, Nat., 364:352-355 (1993); and Wang, J. Cell Biochem., 45:49-53 (1991)), an antisense RNA (Magrath; Ann. Oncol., 5(Suppl 1):67-70 (1994); Milligan et al, Ann. NY Acad. Sci., 716:228-241 (1994); and, Schreier, Pharma. Acta Helv., 68:145-159 (1994)), a catalytic RNA (Cech, Biochem. Soc. Trans., 21:229-234 (1993); Cech, Gene, 135:33-36 (1993); Long et al, FASEB J., 7:25-30; and Rosi et al, Pharm. Therap., 50:245-254 (1991)), or any other sequence which can be transcribed into RNA.

Due to a need for eukaryotic expression cassettes to study the biology of eukaryotic cells, a number of eukaryotic expression plasmids are now readily available. These include, but are not limited to, commercial products from companies such as Invitrogen Corporation (San Diego, CA), Stratagene (La Jolla, CA), ClonTech (Palo

Alto, CA) and Promega Corporation (Madison, WI). All of these plasmids contain the elements of a eukaryotic expression cassette listed above, and many also contain a prokaryotic promoter sequence such that the gene placed downstream from the promoter sequences will be expressed in a prokaryotic as well as in a eukaryotic cell, e.g., plasmid pSV β -gal

contains the eukaryotic SV40 promoter and the prokaryotic gpt promoter which allows for the expression of the β -galactosidase gene in either prokaryotic cells or eukaryotic cells (Promega Corp.).

III. Commercial Applications for the Delivery of Eukaryote Expression Cassettes to Animal Cells

The commercial applications of DNA delivery technology to animal cells are extremely broad and includes delivery of vaccine antigens (Fynan et al, Proc. Natl. Acad. Sci., USA, 90:11478-11482 (1993); Katsumi et al, Hum Gene Ther, 5(11):1335-1339 (1994); and Spooner et al, Int J. Oncol, 6(6):1203-1208 (1995)), immunotherapeutic agents (Shillitoe et al, Eur J Cancer 30B(3):143-154 (1994); Hengge et al, Nature Genetics, 10(2):161-166 (1995); Vile and Hart, Ann Oncol, 5(Suppl 4):59-65 (1994); Miller et al, Ann Surg Oncol, 1(5):436- 450 (1994); and Foa, Baillieres Clin Haematol, 7(2):421- 434 (1994)), and gene therapeutic agents (Darris et al, Canc., 74(3 Suppl.):1021-1025 (1994); Magrath, Ann. Oncol., 5(Suppl 1):67-70 (1994); Milligan et al, Ann. NY Acad. Sci., 716:228-241 (1994) ; Schreier, Pharma. Acta Helv., 68:145-159 (1994); Cech, Biochem. Soc. Trans., 21:229-234 (1993); Cech, Gene, 135:33-36 (1993); Long et al, FASEB J., 7:25-30 (1993); Dropulic and Jeang, Hum. Gene. Ther. , 5:927-939 (1994); Sorscher et al, Hum.

Gene. Ther., 5:1259-1277 (1994); Long et al, FASEB J., 7:25-30 (1993); Nabel et al, Hum. Gene. Ther. 5:89-109 (1994); Manthorpe et al, Hum. Gene. Ther., 4:419-431 (1993); Mittal et al, Virus Res., 28:67-90 (1993); Setoguchi et al, Am. J. Resp. Cell Mol. Biol., 10:369-377 (1994); and Rosi et al, Pharm. Therap., 50:245-254 (1991)).

Significant efforts have been directed to delivery of eukaryotic expression cassettes to animal tissue for gene therapy (Nabel, Circulation, 91:541-548 (1995); Coover et al, Curr. Opin. Neuro., 7:463-470 (1994); Foa, Biol. Clin. Haemat., 7:421-434 (1994) Bowers et al, J. Am. Diet. Assoc., 95:53-59 (1995); Perales et al, Eur. J. Biochem., 226:255-266 (1994); Danko et al, Vacc., 12:1499-1502 (1994); Conry et al, Canc. Res., 54:1164-1168(1994); Ledley, Clin. Invest. Med., 16:78-88 (1993); and Smith, J. Hemat., 1:155-166 (1992)). Gene therapy strategies include genetic complementation of genetic disorders (Caplen et al., Nat. Med., 1:39-46 (1995)), expression of antisense mRNA that specifically blocks the expression of viral genes (Dropulic and Jeang, Hum. Gene Thera., 5:927-939 (1994); Tung and Daniel, Arch. Virol., 133:407-421 (1993)), oncogenes (Fujiwara et al, Curr. Opin. Oncol., 6:96-105 (1994); and Milligan et al, Annal. New York Acad. Sci., 716:228-241 (1994) autoimmune antigens (Steinhoff et al, Proc. Natl. Acad. Sci., USA, 91:5085-5088 (1994) and expression of enzymatically active RNA species to modulate the expression of genes (Altman, Proc. Natl. Acad. Sci., USA, 90:10898-10900 (1993); and Sullivan, J. Invest. Dermatol., 103 (5 Suppl.) :85-89 (1994)).

Significant efforts also have been directed to delivery of eukaryotic expression cassettes to animal tissue for immunotherapy (Pappo et al. J. Surg. Res., 58:218-226 (1995)). Applications in this field include expression of immunoregulatory molecules

such as growth factors or cytokines for tumor therapy (Pardoll, *Curr. Opin. Oncol.*, 4:1124-1129 (1992a); Pardoll, *Curr. Opin. Immunol.*, 4:619-623 (1992b)), expression of tumor-specific antigens for the induction of anti-tumor immunity (Koeppen et al, *Anal. N.Y. Acad. Sci.*, 690:244-255 (1993); and Conry et al., *Gene Ther.*, 2:59-65 (1995)), and delivery of genetic elements that specifically down-regulate host rejection response prior to and following tissue transplantation (Qin et al., *Annal. Surg.*, 220:518-519 (1994)).

"Naked" or polynucleotide DNA vaccines encoding eukaryotic expression cassettes have been used to successfully immunize against influenza both in chickens (Robinson et al, *supra*) and ferrets (Webster et al, *Vacc.*, 12:1495-1498 (1994)); against *Plasmodium yoelii* in mice (Hoffman et al, *supra*); against rabies in mice (Xiang et al, *supra*); against human carcinoembryonic antigen in mice (Conry et al, *supra*) and against hepatitis B in mice (Davis et al, *supra*).

IV. Delivery of Eukaryotic Expression Cassettes to Plant Cells

The use of *Agrobacterium tumefaciens* Ti plasmid to stably deliver DNA to plant cells has been a fundamental advance in plant biotechnology. This system is unique in that it uses a pilus-like structure which binds to the plant cell via specific receptors, and then through a process that resembles bacterial conjugation, delivers the Ti plasmid-linked DNA to the plant cell (Zambryski, *Ann. Rev. Genet.*, 22:1-30 (1988); Lessl et al, *Call*, 77:321-324 (1994); and Walden et al, *Eur. J. Biochem.*, 192:563-576 (1990)). The specificity of this delivery system for plants, however, renders it

ineffective for delivery of DNA to animal cells (Chilton, Proc. Natl. Acad. Sci., USA, 90:3119-3120 (1993); and Walden et al, supra).

V. Delivery of Eukaryotic Expression Cassettes to Animal Cells In Vitro

There are several techniques for introducing DNA into animal cells cultured in vitro. These include the use of cationic liposomes (Hawley-Nelson et al, Focus, 15:73-79 (1993); Ciccarone et al, Focus, 15:80-83 (1993); Pinnaduwaage et al, Biochim. Biophys. Acta, 985:33-37 (1989); Felgner et al, Proc. Natl. Acad. Sci., USA, 84:7413-7417 (1987); Hug and Sleight, Biochim. Biophys. Acta, 1097:1-17 (1991)), chemical methods (Felgner et al, supra; Bothwell et al, Methods for Cloning and Analysis of Eukaryotic Genes, Eds, Jones and Bartlett Publishers Inc., Boston, MA (1990), Ausubel et al, Short Protocols in Molecular Biology, John Wiley and Sons, New York, NY (1992); and Farhood, Annal. N.Y. Acad. Sci., 716:23-34 (1994)), use of protoplasts (Bothwell, supra) electrical pulses (Vatteroni et al, Mutn. Res., 291:163-169 (1993); Sabelnikov, Prog. Biophys. Mol. Biol., 62:119-152 (1994); Brothwell et al, supra; and Ausubel et al, supra), use of attenuated viruses (Morgan et al, supra); Moss, Dev. Biol. Stan., 82:55-63 (1994); and Brothwell et al, supra), and physical methods (Fynan et al, supra; Johnston et al, Meth. Cell Biol, 43 (Pt A) :353-365 (1994); Brothwell et al, supra; and Ausubel et al, supra).

VII. Techniques for the Introduction of Eukaryotic Expression Cassettes into Animal Cells in Vivo

Successful delivery of eukaryotic expression cassettes to animal tissue has been achieved by cationic liposomes (Hazinski et al., Am. J. Resp. Cell Molec. Biol., 4:206-209 (1991); Yoshimura et al., Nuc. Acid Res., 20:3233-3240 (1992); San et al,

Hum. Gene Ther., 4:781-788 (1993) ; Stewart et al, Hum. Gene Ther., 3:267- 275 (1992); Nabel et al, Proc. Natl. Acad. Sci., USA, 90:11307-11311 (1993); Watanabe et al, Mol. Reprod. Dev., 38:268-274 (1994)), avirulent viral delivery vectors such as the retroviral (Belmont et al, Nat., 322:385-387 (1986); Plautz et al., New Biologist, 3:709-715 (1991); Woolf et al., Kidn. Int., 39:S116-119 (1993); Melani et al., Nat. Immun., 13:76-84 (1994); and Kolodka et al., Proc. Natl. Acad. Sci., USA, 92:3293-3297 (1995)) and adenoviral (Engelhardt et al., Nat. Genet., 4:27-34 (1993); Mastrangeli et al., J. Clin. Invest., 91:225-234 (1993); Bajocchi et al., Nat. Genet., 3:229-234 (1993); Curiel, Nat. Immun., 13:141- 164 (1994); Morris et al., J. Urol., 152:506-509 (1994); and Zabner et al., Nat. Genet., 6:75-83 (1994)) delivery systems, direct injection of "naked" DNA into animal muscle tissue or subcutaneously (Robinson et al, Vacc., 11:957-960 (1993); Hoffman et al, Vacc., 12:1529-1533; (1994); Xiang et al, Virol., 199:132-140 (1994); Webster et al, Vacc., 12:1495-1498 (1994); Davis et al, Vacc., 12:1503-1509 (1994); and Davis et al, Hum. Molec. Gen., 2:1847-1851 (1993)), and embryos (Naito et al, Mol. Reprod. Dev., 39:153-161 (1994); and Burdon et al, Mol. Reprod. Dev., 33:436-442 (1992)), and intradermal injection of DNA using "gene gun" technology (Johnston et al, supra).

VIII. Summary

There is a need in the art for an efficient means for delivering eukaryotic expression cassettes including endogenous and/or foreign genes, encoding vaccine, gene therapeutic, or immunotherapeutic agents, to animal cells or tissue.

The present invention describes a novel and unexpected finding that bacterial blebs are capable of delivering eukaryotic expression cassettes to animal cells. An important

aspect of using bacterial blebs to deliver eukaryotic expression cassettes is that they are capable of delivering DNA to mucosal sites.

Heretofore, there has been no documented demonstration of bacterial blebs for introducing eukaryotic expression cassettes, which then are expressed by the treated cells and progeny thereof. That is, the present invention provides the first documentation of genetic exchange between bacterial blebs and animal cells. Heretofore, foreign antigen delivery by live bacterial vector vaccines merely involved delivery of prokaryotic expression cassettes to and expression of the foreign antigen by the bacterial vaccine vector, in animal cells or tissues. In contrast, the present invention involves the delivery of eukaryotic expression cassettes by bacterial blebs to animal cells in vitro or to cells in animal tissue, and expression of the eukaryotic expression cassettes by the animal cell or cells in animal tissue.

SUMMARY OF THE INVENTION

One object of the present invention is to use bacterial blebs to deliver one or more eukaryotic expression cassettes to animal cells or animal tissue.

Another object of the present invention is to use bacterial blebs to deliver one or more eukaryotic expression cassettes encoding vaccine antigen(s) to animal cells or animal tissue.

Another object of the present invention is to use bacterial blebs to deliver one or more eukaryotic expression cassettes encoding gene therapeutic agents to animal cells or animal tissue.

Another object of the present invention is to use bacterial blebs to deliver one or more eukaryotic expression cassettes encoding immunoregulatory agents to animal cells or animal tissue.

Yet another object of the present invention is to use bacterial blebs to deliver one or more eukaryotic expression cassettes encoding biologically active RNA species to animal cells or animal tissue.

In one aspect, the invention relates to a method for introducing and expressing a gene in animal cells, comprising infecting said animal cells with bacterial blebs, wherein the bacterial blebs contain a eukaryotic expression cassette including such gene.

In another aspect, the present invention relates to bacterial blebs, which contain one or more eukaryotic expression cassettes including an endogenous or foreign gene.

Other aspects, features, objects and embodiments of the present invention will be more fully apparent from the ensuing detailed description of the invention.

DETAILED DESCRIPTION OF THE INVENTION

The disclosure of U.S. Provisional Patent Application No. 60/206,994 filed May 24, 2000 in the names of Robert J. Powell and David M. Hone for "Method for Inducing Nucleic Acids into Cells Using Bacterial Blebs as Vectors," is hereby incorporated herein by reference in its entirety.

The present invention in one aspect relates to a method for introducing and expressing a gene in animal cells comprising treating said animal cells with bacterial blebs, wherein the bacterial blebs contain a eukaryotic expression cassette encoding the gene.

Animal cells are defined as nucleated, non-chloroplast containing cells derived from or present in multicellular organisms whose taxonomic position lies within the kingdom animalia. The cells may be present in the intact animal, a primary cell culture, explant culture or a transformed cell line. The particular tissue source of the cells is not critical to the present invention.

The recipient animal cells employed in the present invention are not critical thereto and include cells present in or derived from all organisms within the kingdom animalia, such as those of the families mammalia, pisces, avian, reptilia.

Preferred animal cells are mammalian cells, such as humans, bovine, ovine, porcine, feline, buffalo, canine, goat, equine, donkey, deer, and primate cells. The most preferred animal cells are human cells.

Examples of transformed human cell lines include but are not limited to ATCC Nos. CCL 62, CCL 159, HTB 151, HTB 22, CCL 2, CRL 1634, CRL 8155, HTE 61, and HTBIO4.

Examples of transformed bovine cell lines include ATCC Nos. CRL 6021, CRL 1733, CRL 6033, CRL 6023, CCL 44 and CRL 1390.

Examples of transformed ovine cells lines include ATCC Nos. CRL 6540, CRL 6538, CRL 6548 and CRL 6546.

Examples of transformed porcine cell lines include ATCC Nos. CL 184, CRL 6492, and CRL 1746.

Examples of transformed feline cell lines include CRL 6077, CRL 6113, CRL 6140, CRL 6164, CCL 94, CCL 150, CRL 6075 and CRL 6123.

Examples of transformed buffalo cell lines include CCL 40 and CRL 6072.

Examples of transformed canine cells include ATCC Nos. CRL 6213, CCL 34, CRL 6202, CRL 6225, CRL 6215, CRL 6203 and CRL 6575.

Examples of transformed goat derived cell lines include ATCC No. CCL 73 and ATCC No. CRL 6270.

Examples of transformed horse derived cell lines include ATCC Nos. CCL 57 and CRL 6583.

Examples of transformed deer cell lines include ATCC Nos. CRL 6193-6196.

Examples of transformed primate derived cell lines include those from chimpanzees such as ATCC Nos. CRL 6312, CRL 6304, and CRL 1868; monkey cell lines such as ATCC Nos. CRL 1576, CCL 26, and CCL 161; orangutan cell line ATCC No. CRL 1850; and gorilla cell line ATCC No. CRL 1854.

As used herein, "bacterial blebs" are bacterial membrane-bound vesicles that are capable of delivering eukaryotic expression cassettes to animal cells or animal tissue. "Bacterial blebs" are produced by a number of different bacterial genera including bacteria that are naturally capable of entering the animal cells, as well as bacteria that are not capable of entering animal cells or cells in animal tissue (Dorward and Garon, *supra*).

The particular naturally occurring bleb-producing bacteria employed in the practice of the present invention is not critical thereto. Examples of such naturally occurring bleb-producing bacteria include, but are not limited to, *Shigella* spp., *salmonella* spp., *Neisseria* spp., *Haemophilus* spp. and *Escherichia* spp.

Concerning the use of *Shigella* spp. as a bleb-producing bacterium, the particular *Shigella* strain employed is not critical to the practice of the present invention. Examples of *Shigella* strains that can be employed in the present invention include

Shigella flexneri 2a (ATCC No. 29903), *Shigella sonnei* (ATCC No. 29930), and *Shigella dysenteriae* (ATCC No. 13313). An attenuated *Shigella* strain, such as *Shigella flexneri* 2a 2457T Δ aroA Δ virG mutant CVD 1203 (Noriega et al, supra), *Shigella flexneri* M90T Δ icsA mutant (Goldberg et al, Infect. Immun., 62:5664-5668 (1994)), *Shigella flexneri* Y SFL114 aroD mutant (Karnell et al, Vacc., 10:167-174 (1992)) and *Shigella flexneri* Δ aroA Δ aroD mutant (Verma et al, Vacc., 9: 6-9 (1991)), is preferably employed. Alternatively, new attenuated *Shigella* spp. strains can be constructed by introducing an attenuating mutation either singularly or in conjunction with one or more additional attenuating mutations.

Attenuating mutations can be introduced into bacterial pathogens using non-specific mutagenesis either chemically, using agents such as N-methyl-N'-nitro-N-nitrosoguanidine, or using recombinant DNA techniques; classic genetic techniques, such as Tn10 mutagenesis, P22-mediated transduction, λ phage mediated crossover, and conjugational transfer; or site-directed mutagenesis using recombinant DNA techniques. Recombinant DNA techniques are preferable since strains constructed by recombinant DNA techniques are far more defined. Examples of such attenuating mutations include, but are not limited to:

- (i) auxotrophic mutations, such as aro (Hoiseth et al, Nat., 291:238-239 (1981)), gua (McFarland et al, Microbiol. Path., 3:129-141 (1987)), nad (Park et al, J. Bact., 170:3725-3730 (1988)), thy (Nnalue et al, Infect. Immun., 55:955-962 (1987)), and asd (Curtiss, supra) mutations;
- (ii) mutations that inactivate global regulatory functions, such as cya (Curtiss et al, Infect. Immun., 55:3035-3043 (1987)), crp (Curtiss et al (1987), supra), phoP/phoQ (Groisman et al, Proc. Natl. Acad. Sci., USA,

- 86:7077-7081 (1989); and Miller et al, Proc. Natl. Acad. Sci., USA, 86:5054-5058 (1989)), *phoP^c* (Miller et al, J. Bact., 172:2485-2490 (1990)) or *ompR* (Dorman et al, Infect. Immun., 57:2136-2140 (1989)) mutations;
- (iii) mutations that modify the stress response, such as *recA* (Buchmeier et al, Mol. Micro., 7:933-936 (1993)), *htrA* (Johnson et al, Mol. Micro. 5:401-407 (1991)), *htpR* (Neidhardt et al, Biochem. Biophys. Res. Com., 100:894-900 (1981)), *hsp* (Neidhardt et al, Ann. Rev. Genet., 18:295-329 (1984)) and *groEL* (Buchmeier et al, Sci., 248:730-732 (1990)) mutations;
- (iv) mutations in specific virulence factors, such as *lsyA* (Libby et al, Proc. Natl. Acad. Sci., USA, 91:489-493 (1994)), *pag* or *prg* (Miller et al (1990), supra; and Miller et al (1989), supra), *iscA* or *virG* (d'Hauteville et al, Mol. Micro., 6:833-841 (1992)), *plcA* (Mengaud et al, Mol. Microbiol., 5:367-72 (1991); Camilli et al, J. Exp. Med., 173:751-754 (1991)), and *act* (Brundage et al, Proc. Natl. Acad. Sci., USA, 90:11890-11894 (1993)) mutations;
- (v) mutations that affect DNA topology, such as *topA* (Galan et al, Infect. Immun., 58:1879-1885 (1990)) mutation;
- (vi) mutations that modify or block biogenesis of surface polysaccharides, such as *htrB* (Karow et al, J. Bact., 173:741-750 (1991)), *rfb* or, *galE* (Hone et al, J. Infect. Dis., 156:164-167 (1987) or *via* (Popoff et al, J. Gen. Microbiol., 138:297-304 (1992)) mutations;
- (vii) mutations that modify suicide systems, such as *sacB* (Recorbet et al, App. Environ. Micro., 59:1361-1366 (1993); Quandt et al, Gene,

127:15-21 (1993)), *nuc* (Ahrenholtz et al, *App. Environ. Micro.*, 60:3746-3751 (1994)), *hok*, *gef*, *kil*, or *phlA* (Molin et al, *Ann. Rev. Microbiol.*, 47:139-166 (1993)) mutations;

(viii) mutations that introduce suicide systems, such as lysogens encoded by P22 (Remell et al, *Virol.*, 143:280-289 (1985)), λ murein transglycosylase (Bienkowska-Szewczyk et al, *Mol. Gen. Genet.*, 184:111-114 (1981)) or *S-* gene (Reader et al, *Virol.*, 43:623-628 (1971)); and

(ix) mutations that disrupt or modify the correct cell cycle, such as *minB* (de Boer et al, *supra*) mutation.

The attenuating mutations can be either constitutively expressed or under the control of inducible promoters, such as the temperature sensitive heat shock family of promoters (Neidhardt et al, *supra*), or the anaerobically induced *nirB* promoter (Harborne et al, *Mol. Micro.*, 6:2805-2813 (1992)) or repressible promoters, such as *uapA* (Gorfinkiel et al, *J. Biol. Chem.*, 268:23376-23381 (1993)) or *gcv* (Stauffer et al, *J. Bact.*, 176:6159-6164 (1994)).

With respect to use of *Salmonella* spp. as the bleb-producing bacterium, the particular *Salmonella* strain employed is not critical to the practice of the present invention. Examples of *Salmonella* strains that can be employed in the present invention include *Salmonella typhi* (ATCC No. 7251) and *S. typhimurium* (ATCC No. 13311). Attenuated *Salmonella* strains are preferably used in the practice of the present invention and include *S. typhi* Δ aroA Δ aroD (Hone et al, *Vacc.*, 9:810-916 (1991)) and *S. typhimurium* aroA mutant (Mastroeni et al, *Micro. Pathol.*, 13:477-491 (1992)).

Alternatively, new attenuated *Salmonella* strains can be constructed by introducing one or more attenuating mutations as described for *Shigella* spp. above.

Concerning the use of *Neisseria* spp. as the bleb-producing bacterium, the particular *Neisseria* strain employed is not critical in the broad practice of the present invention. Examples of *Neisseria* strains that can be employed in the present invention include *N. meningitidis* (ATCC No. 13077) and *N. gonorrhoeae* (ATCC No. 19424). Attenuated *Neisseria* strains, such as *N. gonorrhoeae* MS11 aro mutant (Chamberlain et al, Micro. Path., 15:51-63 (1993)) are preferably used in the practice of the present invention. Alternatively, new attenuated *Neisseria* strains can be constructed by introducing one or more attenuating mutations as described for *Shigella* spp. above.

In respect of the use of *Haemophilus* spp. as the bleb-producing bacterium, the particular *Hemophilus* strain employed is not critical in the broad practice of the present invention. Examples of *Hemophilus* strains that can be employed in the practice of the present invention include *H. somnus* (ATCC No. 43625). Attenuated *Hemophilus* strains are preferably used in the practice of the present invention, and can be constructed by introducing one or more attenuating mutations as described for *Shigella* spp. above.

Concerning use of *Escherichia* spp. as the bleb-producing bacterium, the particular *Escherichia* strain employed is not critical in the broad practice of the present invention. Examples of *Escherichia* strains that can be employed in the practice of the present invention include *Escherichia coli* strains 4608-58, 1184-68, 53638-C-17, 13-80, and 6-81 (Sansonetti et al, Ann. Microbiol. (Inst. Pasteur),

132A:351-355 (1982)), *E. coli* H10407 (Elinghorst et al, *Infect. Immun.*, 60:2409-2417 (1992)), and *E. coli* EFC4, CFT325 and CPZ005 (Donnenberg et al, *J. Infect. Dis.*, 169:831-838 (1994)). Attenuated *Escherichia* strains, such as the attenuated turkey pathogen *E. coli* 02 carAB mutant (Kwaga et al, *Infect. Immun.*, 62:3766-3772 (1994)) are preferably used in the practice of the present invention. Alternatively, new attenuated *Escherichia* strains can be constructed by introducing one or more attenuating mutations as described for *Shigella* spp. above.

Additionally, a naturally occurring bleb-forming bacteria or a non bleb-forming bacterial species can be genetically altered to increase or initiate production of blebs. Genetic alterations for altering the bleb-forming ability of a bacterial species include, but are not limited to, deletions in or alterations to genes involved in cell wall formation, septation and the processes of bacterial division. Examples of such genes include, but are not limited to, the outer membrane lipoprotein genes such as *Escherichia coli* mlpD gene (Lange and Hengge-Aronis, *Molec. Microbiol.*, 13:733-743 (1994)), *Bacillus subtilis* LplA gene (Gomez et al, *Microbiol.*, 140:1839-1845 (1994)), *Haemophilus somnus* lppB gene (Theisen et al, *Infect. Immun.*, 61:1793-1798 (1993)), the mini-cell producing genes such as *E. coli* minABDC (Adler et al., supra; and de Boer et al, supra), the *E. coli* htrB (Karow et al, supra), msbB (Karow and Georgopolous, supra), pbpA and pbpB genes (Begg and Donachie, supra); and the *E. coli* cytoplasmic membrane protein FtsL (Guzman et al, *J. Bact.*, 174:7716-7728 (1992)). Such alterations to the genetic makeup of a bacterial strain can be made singularly or in conjunction with other alterations, by any of the methods described above for *Shigella* spp., to any of the bacterial strains listed above or to any other gram negative bacterial species. Bacterial species that are not known to produce

blebs, or do not produce blebs at high rates, but can be altered to either produce blebs or increase their bleb production include, but are not limited to, *Yersinia* spp., *Klebsiella* spp., *Bordetella* spp., *Aeromonas* spp., *Francisella* spp., *Corynebacterium* spp., *Citrobacter* spp., *Chlamydia* spp., *Brucella* spp., *Mycobacterium* spp., *Legionella* spp., *Rhodococcus* spp., *Pseudomonas* spp., *Helicobacter* spp., and *Vibrio* spp.

Concerning use of *Yersinia* spp., the particular *Yersinia* strain employed is not critical in the broad practice of the present invention. Examples of *Yersinia* strains that can be employed in the present invention include *Y. enterocolitica* (ATCC No. 9610) or *Y. pestis* (ATCC No. 19428). Attenuated *Yersinia* strains, such as *Y. enterocolitica* YeO3-R2 (al-Hendy et al, *Infect. Immun.*, 60:870-875 (1992)) or *Y. enterocolitica* aroA (O'Gaora et al, *Micro. Path.*, 9:105-116 (1990)) are preferably used in the practice of the present invention. Alternatively, new attenuated *Yersinia* strains can be constructed by introducing one or more attenuating mutations as described for *Shigella* spp. above.

In respect of use of *Klebsiella* spp., the particular *Klebsiella* strain employed is not critical in the broad practice of the present invention. Examples of *Klebsiella* strains that can be employed in the present invention include *K. pneumoniae* (ATCC No. 13884). Attenuated *Klebsiella* strains are preferably used in the practice of the present invention, and can be constructed by introducing one or more attenuating mutations as described for *Shigella* spp. above.

Concerning the use of *Bordetella* spp., the particular *Bordetella* strain employed is not critical in the broad practice of the present invention. Examples of *Bordetella* strains

that can be employed in the present invention include *B. bronchiseptica* (ATCC No. 19395). Attenuated *Bordetella* strains are preferably used in the practice of the present invention, and can be constructed by introducing one or more attenuating mutations as described for *Shigella* spp. above.

Regarding use of *Aeromonas* spp., the particular *Aeromonas* strain employed is not critical in the broad practice of the present invention. Examples of *Aeromonas* strains that can be employed in the broad practice of the present invention include *A. eucrenophila* (ATCC No. 23309). Alternatively, new attenuated *Aeromonas* strains can be constructed by introducing one or more attenuating mutations as described for *Shigella* spp. above.

In respect of *Franciesella* spp., the particular *Franciesella* strain employed is not critical in the broad practice of the present invention. Examples of *Franciesella* strains that can be employed in the practice of the present invention include *F. tularensis* (ATCC No. 15482). Attenuated *Franciesella* strains are preferably used in the practice of the present invention, and can be constructed by introducing one or more attenuating mutations as described for *Shigella* spp. above.

Concerning *Corynebacterium* spp., the particular *Corynebacterium* strain employed is not critical in the broad practice of the present invention. Examples of *Corynebacterium* strains that can be employed in the present invention include *C. pseudotuberculosis* (ATCC No. 19410). Attenuated *Corynebacterium* strains are preferably used in the practice of the present invention, and can be constructed by introducing one or more attenuating mutations as described for *Shigella* spp. above.

Concerning use of *Citrobacter* spp., the particular *Citrobacter* strain employed is not critical in the broad practice of the present invention. Examples of *Citrobacter* strains which can be employed in the present invention include *C. freundii* (ATCC No. 8090). Attenuated *Citrobacter* strains are preferably used in the practice of the present invention, and can be constructed by introducing one or more attenuating mutations as described for *Shigella* spp. above.

Regarding *Chlamydia* spp., the particular *Chlamydia* strain employed is not critical in the broad practice of the present invention. Examples of *Chlamydia* strains that can be employed in the present invention include *C. pneumoniae* (ATCC No. VR1310). Attenuated *Chlamydia* strains are preferably used in the practice of the present invention, and can be constructed by introducing one or more attenuating mutations as described for *Shigella* spp. above.

In respect of *Brucella* spp., the particular *Brucella* strain employed is not critical in the broad practice of the present invention. Examples of *Brucella* strains that can be employed in the present invention include *B. abortus* (ATCC No. 23448). Attenuated *Brucella* strains are preferably used in the practice of the present invention, and can be constructed by introducing one or more attenuating mutations as described for *Shigella* spp. above.

With respect to *Mycobacterium* spp., the particular *Mycobacterium* strain employed is not critical in the broad practice of the present invention. Examples of *Mycobacterium* strains that can be employed in the present invention include *M. intracellulare* (ATCC

No. 13950) and *M. tuberculosis* (ATCC No. 27294). Attenuated *Mycobacterium* strains are preferably used in the practice of the present invention, and can be constructed by introducing one or more attenuating mutations as described for *Shigella* spp. above.

Concerning *Legionella* spp., the particular *Legionella* strain employed is not critical to the present invention. Examples of *Legionella* strains that can be employed in the present invention include *L. pneumophila* (ATCC No. 33156). Attenuated *Legionella* strains, such as a *L. pneumophila* mip mutant (Ott, FEMS Micro. Rev., 14:161-176 (1994)) are preferably used in the practice of the present invention. Alternatively, new attenuated *Legionella* strains can be constructed by introducing one or more attenuating mutations as described for *Shigella* spp. above.

Concerning *Rhodococcus* spp., the particular *Rhodococcus* strain employed is not critical in the broad practice of the present invention. Examples of *Rhodococcus* strains that can be employed in the present invention include *R. equi* (ATCC No. 6939). Attenuated *Rhodococcus* strains are preferably used in the practice of the present invention, and can be constructed by introducing one or more attenuating mutations as described for *Shigella* spp. above.

The particular *Pseudomonas* strain employed in use of *Pseudomonas* spp. is not critical in the broad practice of the present invention. Examples of *Pseudomonas* strains that can be employed in the present invention include *P. aeruginosa* (ATCC No. 23267). Attenuated *Pseudomonas* strains are preferably used in the present

invention, and can be constructed by introducing one or more attenuating mutations as described for *Shigella* spp. above.

In respect of *Helicobacter* spp., the particular *Helicobacter* strain employed is not critical to the present invention. Examples of *Helicobacter* strains that can be employed in the present invention include *H. mustelae* (ATCC No. 43772). Attenuated *Helicobacter* strains are preferably used in the practice of the present invention, and can be constructed by introducing one or more attenuating mutations as described for *Shigella* spp. above.

When *Vibrio* spp. is employed, the particular *Vibrio* strain utilized is not critical. Examples of *Vibrio* strains that can be employed in the present invention include *Vibrio cholerae* (ATCC No. 14035) and *Vibrio cincinnatiensis* (ATCC No. 35912). Attenuated *Vibrio* strains are preferably used in the practice of the present invention and include *V. cholerae* RSI virulence mutant (Taylor et al, J. Infect. Dis., 170:1518-1523 (1994)) and *V. cholerae* ctxA, ace, zot, cep mutant (Waldor et al, J. Infect. Dis., 170:278-283 (1994)). Alternatively, new attenuated *Vibrio* strains can be constructed by introducing one or more attenuating mutations as described for *Shigella* spp. above.

Alternatively bacterial blebs can be encouraged to form through the treatment of bacterial cells with bacteriocidal and bacteriostatic agents. Examples of chemical induced bleb formation include gentamicin treatment of *Pseudomonas aeruginosa* (Kadurugamuwa et al., supra) and penicillin treatment of *Haemophilus influenzae* (Dargis et al, supra).

As discussed above, the recipient animal cells to which bacterial blebs deliver a eukaryotic expression cassette may be animal cells derived from fish, birds or reptiles.

Examples of bacteria that bind to and naturally interact with fish cells include, but are not limited to *Aeromonas salmonicida* (ATCC No. 33658) and *Aeromonas schubertii* (ATCC No. 43700). Attenuated bacteria are preferably used in the practice of the invention, and include *A. salmonicida* vapA (Gustafson et al, J. Mol. Biol., 237:452-463 (1994) or *A. salmonicida* aromatic-dependent mutant (Vaughan et al, Infect. Immun., 61:2172-2181 (1993)).

Examples of bacteria that naturally interact with avian cells include, but are not restricted to, *Salmonella galinarum* (ATCC No. 9184), *Salmonella enteritidis* (ATCC No. 4931) and *Salmonella typhimurium* (ATCC No. 6994). Attenuated bacteria are preferred in the practice of the invention and include attenuated *Salmonella* strains such as *S. galinarum* cya crp mutant (Curtiss et al, (1987) supra) or *S. enteritidis* aroA aromatic-dependent mutant CVL30 (Cooper et al, Infect. Immun., 62:4739-4746 (1994)).

Examples of bacteria that naturally interact with reptilian cells include, but are not restricted to, *Salmonella typhimurium* (ATCC No. 6994). Attenuated bacteria are preferable in the broad practice of the invention and include attenuated strains such as *S. typhimurium* aromatic-dependent mutant (Hormaeche et al, supra).

The specific animal cells transfected with the bacterial blebs are not critical in the broad practice of the present invention, but will usually be chosen depending on the natural range of cells with which the bacteria, from which the blebs are derived, normally interact.

For example, *Shigella flexneri* naturally interacts with human and primate cells, but rarely dogs and other animals (Kreig et al, *Bergey's Manual of Systematic Bacteriology*, Eds., Wilkins and Williams, Baltimore, MD (1984)), while *Aeromonas salmonicida* interacts with salmonid cells (Austin et al, *Bacterial Fish Pathogens: Diseases in Farmed and Wild Fishes*, Eds., Ellis Harwood Ltd., London (1987)).

The mucosal and systemic immune systems are compartmentalized (Mesteky, *J. Clin. Immunol.*, 7:265-270 (1987); Newby, In: *Local Immune Response of the Gut*, Boca Raton, CRC Press, Newby and Stocks Eds., pages 143- 160 (1984); and Pascual et al., *Immuno. Methods.*, 5:56- 72 (1994)). Thus, antigens delivered to mucosal surfaces elicit mucosal and systemic responses, whereas parentally delivered antigens elicit mainly systemic responses but only stimulate poor mucosal responses (Mesteky, *supra*). Moreover, mucosal stimulation at one mucosal site (for example the intestine) can result in development of immunity at other mucosal surfaces (for example genital/urinary tract) (Mesteky, *supra*). This phenomenon is referred to as the common mucosal system and is well documented (Mesteky, *supra*; and Pascual et al, *supra*).

The development of mucosal vaccines has been hindered by the poor immunogenicity of antigens when delivered by these routes. In this context, antigens can be divided

into two classes: those that bind to intestinal surfaces and those that do not bind, where the former are significantly more immunogenic than the latter (De Aizpurua et al, J. Exp. Med., 176:440-451 (1988). Similarly, delivery of DNA molecules to mucosal surfaces is inefficient due to the many natural host defenses found at these surfaces, such as the gastric barrier and nucleases in the gastrointestinal tract, and the thick mucous layer in the respiratory tract.

Bacterial vectors circumvent these natural barrier functions of the host and enable access to the mucosal compartment (Curtiss, in: New Generation Vaccines: The Molecular Approach, Ed., Marcel Dekker, Inc., New York, NY, pages 161-188 and 269-288 (1989); and Mims et al, in: Medical Microbiology, Eds., Mosby-Year Book Europe, Ltd., London (1993)). Certain enteric and respiratory pathogens, for example, E. coli, Shigella, Listeria, Bordetella and Salmonella, are naturally adapted for this application, as these organisms possess the ability to attach to and invade host mucosal surfaces (Kreig et al, supra). The present invention enables such mucosal compartment access to be exploited, e.g., by oral, intranasal, intravaginal, intrarectal, etc. delivery of bacterial blebs that are engineered to carry eukaryotic expression cassettes to the host mucosal compartment.

Alternatively, any bacteria could be genetically engineered to mimic mucosal tissue tropism properties, as discussed above, that thereby allow bacterial blebs made from such bacterial strain to interact with mucosal tissue, and deliver genes at those sites.

It is also possible to change the tissue specificity of the bacterial blebs by expression of a gene product, in their native form as genetic fusions or truncated versions,

singularly or in combination, e.g., the *Plasmodium vivax* reticulocyte binding proteins-1 and -2 bind specifically to erythrocytes in humans and primates (Galinski et al, Cell, 69:1213-1226 (1992)); *Yersinia* invasin recognizes β 1-integrin receptors (Isberg et al, Trends Microbiol., 2:10-14 (1994)); asialoorosomucoid is a ligand for the asialoglycoprotein receptor on hepatocytes (Wu et al. J. Biol. Chem., 263:14621-14624 (1998)); presence of insulin-poly-L-lysine has been shown to target plasmid uptake to cells with an insulin receptor (Rosenkranz et al, Expt. Cell Res., 199:323-329 (1992)); p60 of *Listeria monocytogenes* allows for tropism for hepatocytes (Hess et al, Infect. Immun., 63:2047-2053 (1995)) and *Trypanosoma cruzi* expresses a 60 kDa surface protein which causes specific binding to the mammalian extra-cellular matrix by binding to heparin, heparin sulfate and collagen (Ortega-Barria et al, Cell, 67:411-421 (1991)). These or other molecules that influence the tissue or cell specificity of the bacterial blebs can also be attached to the surface of purified bacterial blebs by any of various suitable chemical or photo methodologies, as will be appreciated by those skilled in the art in the field of the present invention.

The particular eukaryotic cassette employed in the present invention is not critical in the broad practice of the invention, and can for example be selected from any of the numerous commercially available cassettes, such as pCEP4 or pRc/RSV obtained from Invitrogen Corporation (San Diego, CA), pXT1, pSG5, pPbac or pMbac obtained from Stratagene (La Jolla, CA), pPUR or pMAM obtained from ClonTech (Palo Alto, CA), and pSV β -gal obtained from Promega Corporation (Madison, WI), or synthesized either de novo or by adaptation of a publicly or commercially available eukaryotic expression system.

The individual elements within the eukaryotic expression cassette can be derived from multiple sources and may be selected to confer specificity in sites of action or longevity of the cassettes in the recipient cell. Such manipulation of the eukaryotic expression cassette can be done by any standard or otherwise known molecular biology approach.

These cassettes usually are in the form of plasmids, and contain various promoters well-known to be useful for driving expression of genes in animal cells, such as the viral derived SV40, CMV and, RSV promoters or eukaryotic derived b-casein, uteroglobin, β -actin or tyrosinase promoters. The particular promoter is not critical in the broad practice of the present invention, except in the case where the object is to obtain expression in only selective cell types. In such case, the promoter is selected to be one that is only active in the selected cell type. Examples of tissue specific promoters include, but are not limited to, S1- and β -casein promoters, which are specific for mammary tissue (Platenburg et al, Trans. Res., 3:99-108 (1994); and Maga et al, Trans. Res., 3:36-42 (1994)); the phosphoenolpyruvate carboxykinase promoter, which is active in liver, kidney, adipose, jejunum and mammary tissue (McGrane et al, J. Reprod. Fert., 41:17-23 (1990)); the tyrosinase promoter, which is active in melanocytes, lung and spleen cells, but not testes, brain, heart, liver or kidney (Vile and Hart, Canc. Res., 53:962-967 (1993) and Vile et al, Canc. Res., 54:6228-6234 (1994)); the involucrin promoter, which is only active in differentiating keratinocytes of the squamous epithelia (Carroll et al., J. Cell Sci., 103:925-930 (1992)); neuron specific enolase promoter, which is active only in neurons (Anderson et al., Cell. Molec. Neurobiol., 13:503-515 (1993)), human skeletal alpha-actin and troponin 1 promoters, which are developmentally regulated in

a muscle-specific manner (Dahler et al., *Gene*, 145:305-310 (1994)) and the uteroglobin promoter, which is active in lung and endometrium (Helftenbein et al, *Annal. N.Y. Acad. Sci.*, 622:69-79 (1991)).

Alternatively, cell specific enhancer sequences can be used to control expression; for example, human neurotropic papovirus JCV enhancer regulates viral transcription in glial cells alone (Remenick et al, *J. Virol.*, 65:5641-5646 (1991)). Yet another way to control tissue specific expression is to use a hormone responsive element (HRE) to specify the cell lineages in which a promoter will be active; for example, the MMTV promoter requires the binding of a hormone receptor, such as progesterone receptor, to an upstream HRE before it is activated (Beato, *FASEB J.*, 5:2044-2051 (1991); and Truss et al, *J. Steroid Biochem. Mol. Biol.*, 41:241- 248 (1992)).

Additional genetic elements may be included on the plasmid in order to modify its behavior inside the recipient animal cell (Hodgson, *Bio/Technology*, 13:222-225 (1995). Such elements include, but are not limited to, mammalian artificial chromosome elements or elements from the autonomous replicating circular minichromosomes, such as found in DiFi colorectal cancer cells, to allow stable non-integrated retention of the expression cassette (Huxley et al, *Bio/Technology*, 12:586-590 (1994); and Untawale et al, *Canc. Res.*, 53:1630-1636 (1993)), intergrase to direct integration of the expression cassette into the recipient cells chromosome (Bushman, *Proc. Natl. Acad. Sci., USA*, 91:9233-9237 (1994), the inverted repeats from adeno-associated virus to promote non-homologous integration into the recipient cells chromosome (Goodman et al *Blood*, 84:1492-1500 (1994), recA or a restriction enzyme to promote homologous recombination (PCT Patent Publication No.

case of mRNA) into a polypeptide in vitro or in vivo when placed under the control of appropriate regulatory sequences. The coding sequence boundaries include the start codon at the 5' (amino) terminus and the translation stop codon at the 3' (carboxy) terminus. The coding sequence can include, but is not limited to, cDNA from mRNA, genomic DNA sequences, and synthetic DNA sequences. A transcription termination sequence will usually be located 3' to the gene sequence.

The vaccine antigen may be a protein or antigenic fragment thereof from viral pathogens, bacterial pathogens, parasitic pathogens, or an autoantigen. Alternatively, the vaccine antigen may be a synthetic gene, constructed using recombinant DNA methods, which encode antigens or parts thereof from viral, bacterial, parasitic, etc., pathogens. These pathogens can be infectious in humans, domestic animals or wild animal hosts.

The antigen can be any molecule that is expressed by any viral, bacterial, parasitic, etc., pathogen prior to or during entry into, colonization of, or replication in the animal host. The vaccine antigen can also be an autoantigen, i.e., an antigen that is present at some time spatially or developmentally in the recipient animal cell or tissue.

Multiple eukaryotic expression cassettes can be delivered that express any combination of viral, bacterial, parasitic, etc., antigens, or synthetic genes encoding all or parts or any combination of viral, bacterial, parasitic, etc., antigens. Eukaryotic expression cassettes encoding vaccine antigens can also be delivered in conjunction with additional expression cassettes encoding known adjuvants. Examples of

adjuvants include, but are not limited to, IL-12 (Hall, Sci., 268:1432-1434 (1995)) and bacterial lipopolysaccharide or lipid A (Alving, Immunobiol., 187:430-446 (1993)), outer membrane proteins such as ompA. The viral pathogens, from which the viral antigens are derived, include, but are not limited to, Orthomyxoviruses, such as influenza virus; Retroviruses, such as RSV and SIV, Herpesviruses, such as EBV; CMV or herpes simplex virus; Lentiviruses, such as human immunodeficiency virus; Rhabdoviruses, such as rabies; Picornoviruses, such as poliovirus; Poxviruses, such as vaccinia; Rotavirus; and Parvoviruses.

Examples of protective antigens of viral pathogens include the human immunodeficiency virus antigens nef, p24, gp120, gp41, tat, rev, and pol (Nat., 313:277-280 (1985)) and T cell and B cell epitopes of gp120 (Palker et al, J. Immunol., 142:3612-3619 (1989)); the hepatitis B surface antigen (Wu et al, Proc. Natl. Acad. Sci., USA, 86:4726-4730 (1989)); rotavirus antigens, such as VP4 (Mackow et al, Proc. Natl. Acad. Sci., USA, 87:518-522 (1990)) and VP7 (Green et al, J. Virol., 62:1819-1823 (1988)), influenza virus antigens such as hemagglutinin or nucleoprotein (Robinson et al., supra; Webster et al, supra) and herpes simplex virus thymidine kinase (Whitley et al, In: New Generation Vaccines, pages 825-854).

The bacterial pathogens, from which the bacterial antigens are derived, include, but are not limited to, Mycobacterium spp., Helicobacter pylori, Salmonella spp., Shigella spp., E. coli, Rickettsia spp., Listeria spp., Legionella pneumoniae, Pseudomonas spp., Vibrio spp., and Borellia burgdorferi.

WO9322443 (1993); and PCT Patent Publication No. WO9323534-A (1993)) or elements that direct nuclear targeting of the eukaryotic expression cassette (Hodgson, supra; and Lewin, supra). It may be advantageous to encode some of these elements, such as integrase, recA, and restriction enzymes, in prokaryotic expression cassettes, either on the same genetic element as the eukaryotic expression cassette or alternatively on separate genetic elements. The placing of such toxic or deleterious elements under the control of a prokaryotic promoter ensures that these elements will only be transcribed or translated prior to formation of the bacterial bleb and its separation from the parental bacterial cell, while the prokaryotic translational and transcriptional machinery is present in completion and functioning; thus, a finite dose of the element encoded will be delivered to the eukaryotic recipient.

In accordance with the present invention, the bacterial bleb is employed to deliver eukaryotic expression cassettes encoding a gene into an animal cell or animal tissue. The gene may be either a foreign gene or an endogenous gene. As used herein, "foreign gene" means a gene encoding a protein or fragment thereof or anti-sense RNA or catalytic RNA, which is foreign to the recipient animal cell or tissue, such as a vaccine antigen, immunoregulatory agent, or gene therapeutic agent. An "endogenous gene" means a gene encoding a protein or part thereof or anti-sense RNA or catalytic RNA which is naturally present, or could reasonably be expected to be naturally present in the recipient animal cell or tissue under normal developmental conditions.

The gene, or coding sequence, or sequence that encodes the particular protein, is a nucleic acid molecule that is transcribed (in the case of DNA) and translated (in the

Examples of protective antigens of bacterial pathogens include the *Shigella sonnei* form 1 antigen (Formal et al, Infect. Immun., 34:746-750 (1981)); the O-antigen of *V. cholerae* Inaba strain 569B (Forrest et al, J., Infect. Dis. 159:145-146 (1989)); protective antigens of enterotoxigenic *E. coli* such as the CFA/I fimbrial antigen (Yamamoto et al, Infect. Immun., 50:925-928 (1985)) and the nontoxic B-subunit of the heat-labile toxin (Klipstein et al, Infect. Immun., 40:888-893 (1983)); pertactin of *Bordetella pertussis* (Roberts et al, Vacc., 10:43-48 (1992)), adenylate cyclase-hemolysin of *B. pertussis* (Guiso et al, Micro. Path., 11:423-431 (1991)), and fragment C of tetanus toxin of *Clostridium tetani* (Fairweather et al, Infect. Immun., 58:1323-1326 (1990)).

The parasitic pathogens, from which the parasitic antigens are derived, include, but are not limited to, *Plasmodium* spp., *Trypanosome* spp., *Giardia* spp., *Boophilus* spp., *Babesia* spp., *Entamoeba* spp., *Eimeria* spp., *Laishmania* spp., *Schistosoma* spp., *Brugia* spp., *Fasciola* spp., *Dirofilaria* spp., *Wuchereria* spp., and *Onchocerca* spp.

Examples of protective antigens of parasitic pathogens include the circumsporozoite antigens of *Plasmodium* spp. (Sadoff et al, Sci., 240:336-337 (1988)), such as the circumsporozoite antigen of *P. bergerei* or the circumsporozoite antigen of *P. falciparum*; the merozoite surface antigen of *Plasmodium* spp. (Spetzler et al, Int. J. Pept. Prot. Res., 43:351-358 (1994)); the transmission blocking *P. falciparum* gamete surface proteins Pfs230 (Read et al, Para. Immunol., 16:511-519 (1994)), Pfs48/45 (Kochen et al, Mol. Biochem Parasit., 61:59-68 (1993)) and Pfs2400 (Feng et al, J. Expt. Med., 177:273-281 (1993)); the galactose specific lectin of *Entamoeba histolytica* (Mann et al, Proc. Natl. Acad. Sci., USA, 88:3248-3252 (1991)), gp63 of

Leishmania spp. (Russell et al, J. Immunol., 140:1274-1278 (1988)), paramyosin of *Brugia malayi* (Li et al, Mol. Biochem. Parasitol., 49:315-323 (1991)), the triose-phosphate isomerase of *Schistosoma mansoni* (Shoemaker et al, Proc. Natl. Acad. Sci., USA, 89:1842-1846 (1992)); the secreted globin-like protein of *Trichostrongylus colubriformis* (Frenkel et al, Mol. Biochem. Parasitol., 50:27-36 (1992)); the glutathione-S-transferase's of *Frasciola hepatica* (Hillyer et al, Exp. Parasitol., 75:176-186 (1992)), *Schistosoma bovis* and *S. japonicum* (Bashir et al, Trop. Geog. Med., 46:255-258 (1994)); and KLH of *Schistosoma bovis* and *S. japonicum* (Bashir et al, supra).

Examples of autoantigens include, but are not limited to, fertility antigens such as those which are present on the sperm, oocyte or zona pelucida. Development of an immune response to such antigens is known to block the full-term development of gametes and such antigens can therefore be used in fertility control applications. Illustrative fertility blocking antigens include the 51 kDa sperm-specific glycoprotein FA-1 (Naz and Ahamd, Mol. Reprod. Develop., 39:397-408 (1994)); human sperm 34 kDa protein P34H and hamster 26 kDa epididymal sperm protein (Boue et al, Biol. Reprod., 51:577-587 (1994)); rabbit sperm cap plasma membrane 40 kDa surface antigen (Shaha, Mol. Reprod. Develop., 38:393-403 (1994)); human and mouse antigens recognized by anti-human monoclonal antibodies DAN-2, MOU-8 and VAC-4 (Garcia Framis et al, Immunol. Invest. 23:15-24 (1994)); and the sperm/placenta cross reacting antigen STX-10 (Lee et al, J. Reprod. Immunol., 25:249-264 (1993)).

In accordance with one aspect of the present invention, bacterial blebs can be utilized to deliver eukaryotic expression cassettes encoding a therapeutic agent to animal cells or animal tissue. For example, the eukaryotic expression cassettes can encode tumor-specific, transplant, or autoimmune antigens or parts thereof. Alternatively, the eukaryotic expression cassettes can encode synthetic genes, which encode tumor-specific, transplant, or autoimmune antigens or parts thereof.

Examples of tumor specific antigens include prostate specific antigen (Gattuso et al, Human Pathol., 26:123-126 (1995)), TAG-72 and CEA (Guadagni et al, Int. J. Biol. Markers, 9:53-60 (1994)), MAGE-1 and tyrosinase (Coulie et al, J. Immunothera., 14:104-109 (1993)). It has been demonstrated in mice that immunization with non-malignant cells expressing a tumor antigen provides a vaccine effect, and also helps the animal thereby treated to mount an immune response to clear malignant tumor cells displaying the same antigen (Koeppen et al, supra); additionally, vaccination with a human carcinoembryonic antigen vaccine has been demonstrated to protect mice against a challenge by colon carcinoma cells (Conry et al., supra).

Examples of transplant antigens include the CD3 receptor on T cells (Alegre et al, Digest. Dis. Sci., 40:58-64 (1995)). Treatment with an antibody to CD3 receptor has been shown to rapidly clear circulating T cells and reverse most rejection episodes (Alegre et al, supra). It has also been shown that delivery of a gene encoding transforming growth factor β -1 to tissue grafts can prolong allograft survival (Qin et al, supra).

Examples of autoimmune antigens include IAS β -chain (Topham et al, Proc. Natl. Acad. Sci., USA, 91:8005-8009 (1994)). Vaccination with an 18 amino acid peptide from IAS β chain has been demonstrated to provide protection and treatment to mice with experimental autoimmune encephalomyelitis (Topham et al, supra).

In addition, the bacterial blebs described herein can be used to deliver gene therapeutic agents or genes to recipient animal cells or animal tissue. Strategies for gene therapy include the genetic complementation of inherited or spontaneous genetic disorders, mutations, or deficits (Liszewicz, Leuk., 8:S152-155 (1994)), and the supplementation of genes in order to enhance or alter the dose of a particular encoded factor or enzyme. Genetic elements delivered in eukaryotic expression cassettes by bacterial blebs to complement a mutated or non-functional gene in the animal cell can encode an entire replacement gene, or set of related genes, a complimentary DNA sequence encoding a primary RNA transcript, partially or completely processed RNA transcript, trans- or cis- acting regulatory element, enhancer or other modulatory factor. In order to complement some genetic defects it may be necessary to deliver one or more eukaryotic expression cassettes each encoding one or more components of a biochemical pathway, multi-enzyme process, or gene therapeutic elements can be delivered individually or in combination with other genes, or other eukaryotic expression cassettes. Eukaryotic expression cassettes delivered to animal cells by bleb-mediated transfection could be under the control of tissue specific, constitutive and/or inducible promoters, enhancers, or other transcriptional modification systems, as described above.

The advent of increasingly more powerful molecular techniques has recently resulted in an exponential growth of information on genetic lesions and the disease states resulting from such lesions. A large number of genetic lesions and resulting disease states has therefore been identified. Diseases for which a specific genetic lesion has been defined and appertaining in vitro or in vivo treatments reported. The gene or genes in which the genetic lesions occur include, but are not limited to, cystic fibrosis-cystic fibrosis transmembrane conductance regulator (Yoshimura et al., *Nuc. Acids Res.*, 20:3233-3240 (1992); Zabner et al., *Cell*, 75:207-216 (1993); Zabner et al, *supra* (1994); Caplen et al, *supra*); emphysema- α 1 antitrypsin (Setoguchi et al., *Am. J. Resp. Cell. Molec. Biol.*, 10:369-377 (1994)); familial hypercholesterolaemia-LDL receptor (Grossman et al, *Nat. Genet.*, 6:335-341 (1994)); fanconi anemia-fanconi anemia C complementing gene (Walsh et al., *Blood*, 84:453-459 (1994)); hypertension-kallikrein gene (Wang et al., *J. Clin. Invest.*, 95:1710-1716 (1995) mucopolysaccharidosis type II (Hunter syndrome)-iduronate-2-sulfatase (Braun et al., *Proc. Natl. Acad. Sci., USA*, 90:11830-11834 (1993)); propionyl coA carboxylase deficiency-PCCA (Stankovics and Ledley, *Am. J. Hum. Genet.*, 52:144-151 (1993)); Sly syndrome-beta-glucuronidase (Moullier et al, *Nat. Genet.*, 4:154-159 (1993)); and X-linked ichthyosis-steroid sulphatase (Jensen et al., *Exp. Cell Res.*, 209:392-397 (1993)). Addition of a correctly functioning copy of the mutated or non-functional gene, via blebs transfection in accordance with the invention, embodies a useful approach to the treatment of such genetic deficiencies.

As another aspect of the present invention, bacterial blebs can deliver eukaryotic expression cassettes encoding immunoregulatory molecules. These immunoregulatory molecules include, but are not limited to, growth factors, such as

M-CSF, GM-CSF; and cytokines, such as IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13 or IFN- γ . In application to tumor tissue, cytokine expression cassettes can be employed to stimulate potent systemic immunity and enhanced tumor antigen presentation without producing a systemic cytokine toxicity (Golumbek et al, *Canc. Res.*, 53:5841-5844 (1993); Golumbek et al, *Immun. Res.*, 12:183-192 (1993); Pardoll, (1992a) *supra*; Pardoll, (1992b) *supra*; Melani et al, *Nat. Immun.*, 13:76-84 (1994); and Harris et al., *Cancer.*, 74S:1021-1025 (1994)), and cause a reduction in the size of the tumor (Cignetti et al, *J. Natl. Canc. Inst.*, 86:785-791 (1994); Pappo et al, *supra*; and Cordier et al., *Gene Ther.*, 2:16-21 (1995)).

In antisense RNA and catalytic RNA applications, the antisense RNA and catalytic RNA species delivered to animal cells can be targeted against any molecule present within the recipient cell or likely to be present within the recipient cell. These include but are not limited to RNA species encoding cell regulatory molecules, such as interleukin-6 (Mahieu et al, *Blood*, 84:3758-3765 (1994)), oncogenes such as ras (Kashani-Sabet et al, *Antisen Res. Devel.*, 2:3-15 (1992)), causative agents of cancer such as human papillomavirus (Steele et al, *Canc. Res.*, 52:4706-4711, (1992)), enzymes, viral RNA's and pathogen derived RNAs such as HIV-1, (Chuah et al, *Hum. Gene. Thera.*, 15:1467-1475 (1994); Meyer et al, *Gene*, 129:263-268 (1993); Chatterjee et al, *Sci.*, 258:1485-1488 (1992); Tung and Daniel, *supra*; and Yamada et al, *Virol.*, 205:121-126 (1994)). RNAs can also be targeted at non-transcribed DNA sequences, such as promoter or enhancer regions, or to any other molecule present in the recipient cells, such as but not limited to, enzymes involved in DNA synthesis or TRNA molecules (Scanlon et al, *Proc. Natl. Acad. Sci. USA*, 88:10591-10595 (1991); and Baier et al, *Mol. Immunol.*, 31:923-932 (1994)).

In the broad practice of the present invention, bacterial blebs can also be used to deliver eukaryotic expression cassettes encoding proteins to animal tissue from which the expressed proteins can later be harvested or purified. An example is the delivery of a eukaryotic expression cassette under the control of a mammary specific viral promoter, such as derived from mouse mammary tumor virus (ATCC No. VR731), encoding α 1-antitrypsin to mammary tissue of a goat or sheep.

As a further alternative, single or multiple eukaryotic expression cassettes encoding tumor-specific, transplant, and/or autoimmune antigens, can be delivered in any single or multiple combination with eukaryotic expression cassettes encoding immunoregulatory molecules or other proteins.

The invention also encompasses delivery of prokaryotic expression cassettes in combination with eukaryotic expression cassettes.

The bacterial blebs containing the eukaryotic expression cassette can be used to treat animal cells that are cultured in vitro. The animal cells can be further cultured in vitro, and the cells carrying a desired genetic trait can be enriched by selection for or against any selectable marker introduced to the recipient cell at the time of bactofection. Such markers may include antibiotic resistance genes, e.g., hygromycin, or neomycin, selectable cell surface markers, or any other phenotypic or genotypic element introduced or altered by bactofection. These in vitro-infected cells or the in vitro-enriched cells can then be introduced into animals intravenously,

intramuscularly, intradermally, or intraperitoneally, or by any inoculation route that allows the cells to enter the host tissue.

Alternatively, the bacterial blebs containing the eukaryotic expression cassettes can be introduced to infect the animal by intravenous, intramuscular, intradermal, intraperitoneal, peroral, intranasal, intraocular, intrarectal, intravaginal, oral, immersion, intraurethral, or any other suitable administration or inoculation routes.

The amount of the bacterial blebs of the present invention to be administered will vary depending on the species of the subject, as well as the disease or condition that is being treated. Generally, the dosage employed will be in a range of from about 10^2 to about 10^{21} blebs, preferably from about 10^6 to about 10^{12} blebs. Alternatively, when transfecting individual cells in vitro, the dosage of blebs to be administered will vary depending on the cells, but generally the ratio of blebs:cells will be in a range of from about 0.1:1 to about 10^9 :1, and preferably from about 1:1 to about 10^5 :1.

The bacterial blebs of the present invention are generally administered along with a pharmaceutically acceptable carrier or diluent.

The particular pharmaceutically acceptable carrier or diluent employed is not critical in the general practice of the present invention. Examples of diluents include physiological media used for in vitro tissue culture such as RPMI, DMEM or MEM, a phosphate buffered saline, buffer for buffering against gastric acid in the stomach, such as citrate buffer (pH 7.0) containing sucrose, bicarbonate buffer (pH 7.0) alone (Levine et al, J. Clin. Invest., 79:888-902 (1987); and Black et al J. Infect. Dis.,

155:1260-1265 (1987)), or bicarbonate buffer (pH 7.0) containing ascorbic acid, lactose, and optionally aspartame (Levine et al, Lancet, II: 467-470 (1988)). Examples of carriers include proteins, e.g., as found in skim milk or serum, sugars (e.g., sucrose), glycerol, ammonium sulfate or polyvinylpyrrolidone. Typically these carriers are used at a concentration in a range of about 0.1 to about 90% (w/v), and preferably in a range of from about 1 to about 20% (w/v).

The features and advantages of the present invention are more fully shown by the following non-limiting examples, which are provided for illustrative purposes only, and are in no way intended to limit the scope of the present invention.

Example I

Bleb-Mediated Transfection Using a Eukaryotic Expression Cassette

The following experiment was carried out to demonstrate that bacterial blebs are able to introduce a foreign gene into animal cells, which is then expressed by the animal cells (hereinafter "bleb-mediated transfection").

A. The Eukaryotic Expression Cassette

An attenuated *Shigella flexneri* strain containing both Δ aro Δ and Δ virG attenuating lesions (Noriega et al, supra) was transformed with the dual prokaryotic/eukaryotic expression cassette containing plasmid pSV β -gal (Promega Corporation, Madison, WI), which contains the reporter gene β -galactosidase (β -gal) (hereinafter "p β -gal+SV"), under the control of both a prokaryotic (gpt) as well as a viral derived, eukaryotic (SV40) promoter. A second population of the same *Shigella flexneri* strain

containing both Δ aro Δ and Δ virG attenuating lesions (Noriega et al, supra) was transformed with an expression cassette containing containing plasmid p β -gal-SV, a derivative of p β -gal+SV from which the eukaryotic SV40 promoter had been removed by standard molecular biology techniques leaving only a prokaryotic expression cassette. Thus plasmid p β -gal+SV was capable of directing synthesis of β -gal enzyme inside bacteria as well as animal cells, while plasmid p β -gal-SV was capable of directing synthesis of β -gal enzyme only inside bacteria.

The resulting transformed bacteria were seeded from 30% (w/v) glycerol stocks, maintained at -70°C , onto solid medium (Tryptic Soy Agar, DIFCO, Madison, WI) containing 100 mg/ml of ampicillin, to select for bacteria containing the plasmids, and grown overnight at 37°C .

B. Isolation of Bacterial Blebs

Bacterial blebs were prepared from cells grown on solid media (Tryptic soy agar) overnight at 37°C . Cells were either harvested directly from solid media by scraping, or seeded into liquid media (RPMI) to an optical density of 0.2 OD₆₀₀ and grown for three hours before harvesting. Bacterial cells harvested from solid media were suspended in RPMI at a density of approximately 0.1 g cells per mL and mixed vigorously by vortex action for 5 minutes. Whole bacterial cells were pelleted from liquid cultures or bacterial suspensions by centrifugation at 10,000 xg for 10 mins. The supernatant was removed and centrifuged again at 10,000 xq for 20 mins. The resulting cell-free, bleb-containing supernatant was filtered through a 0.2 μm filter then concentrated 10-20 fold with an Amicon (Lexington, MA) stirred cell ultrafiltration cell using a 100k Da cutoff membrane before storage at 4°C . Protein

concentration was determined by BCA assay (Pierce, Rockford IL). Concentrated bleb preparations were confirmed bacterial free by plating 50 μ L on Tryptic Soy Agar, and seeding 2 x 50 μ L into 5 mL RPMI each. If no growth was detected after 24 hours at 37°C, the resultant bleb-fractions were used for transfection assays.

C. The Eukaryotic Cells

HeLa cells (ATCC No. CCL-2) were grown on plastic tissue culture plates at 37°C in 5% (v/v) CO₂ in RPMI medium supplemented with 10% (v/v) fetal bovine serum, 2.0 mM L-glutamine, 1.0 mM L-pyruvate, 50 U/ml penicillin and 50 mg/ml streptomycin (hereinafter "RPMI/FBS"). 24 to 48 hours prior to bactofection, the HeLa cells were trypsinized with 0.25% (w/v) trypsin containing 1.0 mM EDTA, and split by limiting dilution such that they were 40-60% confluent at the time of the experiment.

Prior to bleb-mediated transfection, the number of HeLa cells present was ascertained by counting in a hemocytometer (Celis, Cell Biology: A Laboratory Manual, Ed., Academic Press, San Diego, CA (1994)).

D. Bleb-Mediated Transfection of the Eukaryotic Cells

5×10^4 HeLa cells were washed once with RPMI media lacking fetal bovine serum and penicillin/streptomycin (hereinafter "SFM"), then overlaid with a bleb-containing suspension, and incubated at 37°C in 5% CO₂. The bleb suspension was either off the overnight agar plates or from a fresh broth culture, and was prepared to give an infection ratio of approximately 5 to 100 μ g protein to 1 HeLa cell.

After 3 hours, the bleb-containing media was removed, the HeLa cells were rinsed once with RPMI/FBS, and then fresh RPMI/FBS was added and the cells were returned to 37°C in 5% CO₂.

At 6 hours, 24 hours, 48 hours, and 92 hours post-bleb-mediated transfection, the HeLa cells were washed twice with phosphate buffered saline (hereinafter "PBS"), stained for β -gal activity as described by Hawley-Nelson et al, Focus, 15:73-79 (1993), and then photographed. When the incubation time prior to analysis was greater than 48 hours, then the RPMI/FBS was changed at 48 hours.

Cells in which the cytoplasm had stained evenly blue were only observed for HeLa cells transfected with bleb-containing supernatants derived from Shigella cells in which the p β -gal+SV plasmid had been present, i.e., those blebs which contained a plasmid containing a eukaryotic expression cassette. HeLa cells transfected with blebs derived from Shigella cells in which the p β -gal-SV plasmid had been present did not result in a transfected, evenly staining, β -gal positive phenotype. Thus, transfection of HeLa cells with blebs that contained only a prokaryotic expression cassette did not result in a transformed phenotype. Blebs prepared from both Shigella cell types (i.e., those containing p β -gal+SV, as well as those containing p β -gal-SV) resulted in HeLa cells that showed discrete blue stained 'dots' resulting from the delivery of bacterially synthesized β -gal enzyme, i.e., enzyme synthesized prior to the formation of the bleb and its separation from the bacterial parent cell. None of the HeLa cells transfected with blebs isolated from the Shigella cells containing p β -gal-SV resulted in a transformed phenotype. The average extent of confluent staining was greater the longer the period between bleb-mediated transfection and staining, which

indicates that expression was not due solely to β -gal synthesized by the *Shigella* prior to bleb formation, and packaged in the bleb at the time of formation, but rather was a result of β -gal newly synthesized within the HeLa cell.

The foregoing empirically demonstrates that bacterial blebs can be used to deliver a eukaryotic expression cassette to animal cells for expression therein of the contained genetic information.

Example 2

Blebs delivery of pre-synthesized molecules to animal cells

The results in Example 1 also demonstrated that bacterial blebs are able to deliver pre-synthesized molecules to animal cells, as shown by the ability of blebs derived from *Shigella* in which the p β -gal-SV plasmid had been present. Thus, bacterial blebs can be used to deliver previously synthesized molecules, produced from prokaryotic expression cassettes by the bacteria from which the bleb was derived, prior to bleb formation, to recipient animal cells.

Example 3

Production of non-pyrogenic blebs from *Salmonella*

In this example we show that Blebs can be produced, and purified, from a non-pyrogenic *Salmonella* strain. To produce the Blebs, a non-pyrogenic *Salmonella* strain herein designated "T109", which is a *Salmonella* carrying deletion mutations in

the *aroA* and *msbB* genes, was cultured in 100 ml luria-bertani broth (LB; Difco, Detroit MI) at 37°C with agitation (i.e. 200 oscillations per min; OPM) to an optical density at 600 nm (OD_{600}) of from 0.4 to 0.8 relative to a sterile LB control. At this point, gentamicin (Lifetechnologies; Gaithersburg, MD) is added to a final concentration of 5 µg/ml and the culture is incubated as above for a further 30 min. To harvest the blebs, the bacteria were removed by centrifugation at 7,000 X g for 20 min. Following centrifugation the supernatant, which contains the Blebs, was decanted into a sterile centrifuge tube. The Blebs were harvested from the supernatant by centrifugation at 20,000 X g for 30 min. Following this second centrifugation step, the supernatant was discarded and the pellet, which contained the Blebs, was resuspended in 1 ml endotoxin-free phosphate buffered saline (PBS; Lifetechnologies, Gaithersburg, MD). To remove trace bacteria that remained in the enriched Bleb preparation, this latter 1 ml suspension was passed through a 1.2 µm filter (Nalgene, Rochester NY), then a 0.45 µm filter (Millipore, Bedford MA). To validate sterility 10 µl aliquots of the filtrate were plated onto a series of 5 luria-bertani agar (LA) plates and incubated at 37°C for 16 hr. No colonies appeared following incubation, indicating that the filtrates, which contained the non-pyrogenic *Salmonella* blebs, contained <10 colony forming units (cfu) per ml. To verify that the filtrates contained Blebs, 10 µl of the filtrate was placed onto a microscope slide and examined 400X magnification using a Nikon Eclipse TE300 microscope (Tokyo, Japan). The concentration of Blebs in the filtrates was estimated to be about 10^9 per ml.

To gain better measure of the concentration of Blebs in the suspensions described above, the total protein concentration was determined by BCA quantitative protein assay (Pierce, Rockford, IL). A standard curve was produced using bovine serum albumin (Pierce, Rockford IL) according to the manufacturer's direction. The total protein concentration was found to be 80 ng per ml. In addition, the total lipopolysaccharide concentration was measured using the liminus amebocyte lysate (LAL) assay (Cape Cod Associates, Cape Cod ME). The LAL assay indicated that the LPS concentration in the Bleb preparation was around 100 ng per ml. These values provided a measure to standardize the dose of Blebs for the *in vivo* studies described in the following example.

Example 4

Vaccination of mice with non-pyrogenic *Salmonella* Blebs

Individual groups of three specific pathogen-free, 18-20 gram female BALB/cAnNCrIBR mice (Charles River Laboratories, Wilmington, MA) were vaccinated with a single dose of about 10 pg or 50 pg protein concentration equivalent of T109 Blebs suspended in 100 μ l PBS, by intragastric intubation. Prior to vaccination, the mice were given 100 μ l of 50% (w/v) bicarbonate, also by intragastric intubation.

Prior to and one week after vaccination, about 100 μ l of blood was collected from the tail vein of each mouse and allowed to clot. The sera from these samples was collected by first removing the clots by centrifugation in a microfuge for 5 min at 13,000 rpm and decanting the sera into fresh tubes.

The *Salmonella*-specific serum IgG responses were measured in these sera using a conventional ELISA and whole inactivated *Salmonella* as antigen. Briefly, the wells of a 96 well microtiter plate were treated with 100 μ l of acetone-inactivated *Salmonellae* suspended in PBS at a concentration of 10^7 bacilli per ml for 16 hr at room temperature. These plates were subsequently washed with PBS and 200 μ l of 5% (w/v) non-fat dried milk (suspended in PBS) was added to each well. After a further 1 hr the wells were washed once with 200 μ l PBS and 100 μ l of samples of diluted pre- and post-vaccination sera were added, starting at a dilution of 1:30 and in 3-fold serial dilutions to 1:27,000. The plates were incubated for a further 1 hr and the wells were washed 4 times with 200 μ l of PBS. *Salmonella*-specific IgG was detected using colorimetrically using a 1:1000 dilution of rat anti-mouse IgG-horse-radish peroxidase conjugate (Sigma, St Louis MO) and standard substrates, according to the directions of the manufacturer. The results of the ELISA show that vaccination of mice with a single dose of the non-pyrogenic *Salmonella* Blebs induced a strong serum IgG response against *Salmonella* (Figure 1).

The results show conclusively that vaccination of mice with both the 10 pg and 50 pg doses of non-pyrogenic *Salmonella* Blebs resulted in the development of strong serum IgG responses against *Salmonella*. Since serum IgG responses against *Salmonella* have been shown previously to correlate with protection against *Salmonella*, these data show that non-pyrogenic Blebs are useful and inexpensive method for producing vaccines against bacterial pathogens.

Example 5

Production of non-pyrogenic blebs containing a DNA vaccine

In this example we show that Blebs can be produced by, and purified from, a non-pyrogenic *Salmonella* strain that carries DNA vaccine pOGL1-A1 (Sequence ID #1; Figure 3). Plasmid pOGL1-A1 expresses the 120 kDA glycoprotein (i.e. gp120) of human immunodeficiency virus type 1 (HIV-1) and the A1 subunit of cholera toxin (i.e. CtxA1). We have shown previous that intramuscular vaccination with the dicistronic DNA vaccine pOGL1-A1 induces strong immune responses against HIV-1 (Hone et al, US patent pending). Production of the Blebs from strain H1071, which is a non-pyrogenic *Salmonella* strain SL7207 carrying deletion mutations in the *aroA* and *msbB* genes and harboring DNA vaccine pOGL1-A1, was accomplished by growing H1071 in 100 ml luria-bertani broth (LB; Difco, Detroit MI) at 37°C with agitation (i.e. 200 oscillations per min; OPM) to an optical density at 600 nm (OD₆₀₀) of from 0.4 to 0.8 relative to a sterile LB control. At this point, gentamicin (Lifetechnologies; Gaithersburg, MD) was added to a final concentration of 5µg/ml and the culture was incubated as above for a further 30 min. To harvest the blebs, the live whole bacteria were removed by centrifugation at 7,000 X g for 20 min. Following centrifugation the supernatant containing the Blebs, was decanted into a sterile centrifuge tube. The H1071 Blebs were then harvested from the supernatant by centrifugation at 20,000 X g for 30 min. Following this second centrifugation step, the supernatant was discarded and the pellet containing the H1071 Blebs was resuspended in 1 ml endotoxin-free phosphate buffered saline (PBS; Lifetechnologies, Gaithersburg, MD). As shown in example 1 above, trace bacteria that remained in the enriched Bleb preparation were removed by passing this suspension through a 1.2 µM filter (Nalgene, Rochester NY), then a 0.45 µM filter (Millipore, Bedford MA).

To validate sterility 10 μ l aliquots of the filtrate were plated onto a series of 5 luria-bertani agar (LA) plates and incubated at 37°C for 16 hr. No colonies appeared following incubation, indicating that the filtrates, which contained the non-pyrogenic *Salmonella* blebs, contained <10 colony forming units (cfu) per ml. To verify that the filtrates contained Blebs, 10 μ l of the filtrate was placed onto a microscope slide and examined 400X magnification using a Nikon Eclipse TE300 microscope (Tokyo, Japan). The concentration of Blebs in the filtrates was estimated to be about 10^8 - 10^9 Blebs per ml.

To gain better measure of the concentration of Blebs in the suspensions described above, the total protein concentration was determined by BCA quantitative protein assay (Pierce, Rockford, IL). A standard curve was produced using bovine serum albumin (Pierce, Rockford IL) according to the manufacturer's direction. The total protein concentration was found to be 80 ng per ml. In addition, the total lipopolysaccharide concentration was measured using the liminus amebocyte lysate (LAL) assay (Cape Cod Associates, Cape Cod ME). The LAL assay indicated that the LPS concentration in the Bleb preparation was around 100 ng per ml. These values provided a measure to standardize the dose of Blebs for the *in vivo* studies described in the following example. Presence of pOGL1-A1 in the Bleb preparation was confirmed by polymerase chain reaction amplification of sequences in pOGL1-A1 using conventional procedures well known in the art and primers specific for sequences encoding gp120. The PCR was strongly positive indicating that the Blebs harbored substantial levels of pOGL1-A1 DNA.

Example 6

Vaccination of mice with non-pyrogenic Blebs harboring a DNA vaccine

Individual groups of three specific pathogen-free, 18-20 gram female BALB/cAnNCrIBR mice (Charles River Laboratories, Wilmington, MA) were vaccinated with a single dose of about 10 pg or 50 pg protein concentration equivalent of H1071 Blebs suspended in 100 μ l PBS, by intragastric intubation. Prior to vaccination, the mice were given 100 μ l of 50% (w/v) bicarbonate, also by intragastric intubation. Prior to and one week after vaccination, about 100 μ l of blood was collected from the tail vein of each mouse and allowed to clot. The sera from these samples was collected by first removing the clots by centrifugation in a microfuge for 5 min at 13,000 rpm and decanting the sera into fresh tubes.

The gp120-specific serum IgG responses were measured in these sera using a conventional ELISA and purified recombinant gp120 as antigen. Briefly, the wells of a 96 well microtiter plate were treated with 100 μ l of gp120 suspended in PBS at a concentration of 10 μ g per ml for 16 hr at room temperature. These plates were subsequently washed with PBS and 200 μ l of 5% (w/v) non-fat dried milk (suspended in PBS) was added to each well. After a further 1 hr the wells were washed once with 200 μ l PBS and 100 μ l of samples of diluted pre- and post-vaccination sera were added, starting at a dilution of 1:30 and in 3-fold serial dilutions to 1:27,000. The plates were incubated for a further 1 hr and the wells were washed 4 times with 200 μ l of PBS. Gp120-specific IgG was detected using colorimetrically using a 1:1000 dilution of rat anti-mouse IgG-horse-radish peroxidase conjugate (Sigma, St Louis

MO) and standard substrates, according to the directions of the manufacturer. The results of the ELISA show that vaccination of mice with a single dose of the non-pyrogenic *Salmonella* Blebs harboring POGL1-A1 induced a strong serum IgG response against gp120 (Figure 2).

The results (Figure 2) show conclusively that vaccination of mice with both the 10 pg and 50 pg doses of non-pyrogenic *Salmonella* Blebs harboring POGL1-A1 induced a strong serum IgG response against gp120 resulted in the development of strong serum IgG responses against gp120. These data show that non-pyrogenic Blebs are a useful and inexpensive tool for delivering DNA vaccines to the lymphoid compartment *in vivo* and inducing immune responses against DNA vaccine-encoded DNA vaccines.

While the invention has been described herein with reference to various illustrative features, aspects, and embodiments, it will be appreciated that the utility of the invention is not thus limited, but rather extends to and encompasses other variations, modifications and other embodiments, as will readily suggest themselves to those of ordinary skill in the art. Accordingly, the invention is to be broadly interpreted and construed as including such other variations, modifications and other embodiments, within the spirit and scope of the invention as hereinafter claimed.

THE CLAIMS

1. A method for introducing and expressing a gene in animal cells comprising infecting said animal cells with bacterial blebs, wherein said bacterial blebs contain a eukaryotic expression cassette encoding said gene.
2. The method of Claim 1, wherein said gene is selected from the group consisting of a gene encoding a vaccine antigen, gene therapeutic agent, immunoregulatory agent, antisense RNA, or catalytic RNA.
3. The method of Claim 1, wherein said animal cell is a mammalian cell.
4. The method of Claim 3, wherein said mammalian cell is selected from the group consisting of humans, cattle, sheep, goat, horse, donkey, primate, and buffalo cells.
5. The method of Claim 4, wherein said mammalian cell is human cells.
6. The method of Claim 1, wherein said bacterial blebs are derived from bacteria selected from the group consisting of *Shigella* spp., *Salmonella* spp., *Neisseria* spp., *Haemophilus* spp., *Vibrio* spp., and *Escherichia* spp.

7. The method of Claim 6, wherein said bacteria are attenuated.
8. The method of Claim 1, wherein said bacterial blebs are derived from bacteria selected from the group consisting of *Yersinia* spp., *Escherichia* spp., *Klebsiella* spp., *Bordetella* spp., *Neisseria* spp., *Aeromonas* spp., *Francisella* spp., *Corynebacterium* spp., *Citrobacter* spp., *Chlamydia* spp., *Hemophilus* spp., *Brucella* spp., *Mycobacterium* spp., *Legionella* spp., *Rhodococcus* spp., *Pseudomonas* spp., *Helicobacter* spp., *Bacillus* spp., *Leishmania* spp. and *Erysipelothrix* spp. which have been genetically engineered to mimic the bleb-producing properties of *Shigella* spp., *Salmonella* spp., *Neisseria* spp., *Haemophilus* spp., *Vibrio* spp., and *Escherichia* spp.
9. The method of Claim 8, wherein said bacteria are attenuated.
10. The method of Claim 1, wherein said animal cells are infected with about 10^2 to 10^{21} bacterial blebs.
11. The method of Claim 10, wherein said animal cells are infected with about 10^6 to 10^{12} bacterial blebs.
12. The method of Claim 1, wherein said animal cells are infected at a ratio of blebs to animal cells ranging from about 0.1 to 10^9 .

13. The method of Claim 12, wherein said animal cells are infected at a ratio of blebs to animal cells ranging from about 10^0 to 10^5 .
14. The method of Claim 1, wherein said animals cells are cultured in vitro.
15. The method of Claim 1, wherein said bacterial blebs are administered to an animal.
16. The method of Claim 15, wherein said animal is a mammal.
17. The method of Claim 16, wherein said mammal is a human.
18. The method of Claim 15, wherein said bacterial blebs are intranasally administered.
19. The method of Claim 16 wherein said bacterial blebs are intranasally administered.
20. The method of Claim 17, wherein said bacterial blebs are intranasally administered.
21. A bacterial bleb containing a eukaryotic expression cassette.
22. The bacterial bleb of Claim 21, wherein the eukaryotic expression cassette includes a polynucleotide coding sequence encoding a vaccine

antigen, gene therapeutic agent, immunoregulatory agent, antisense RNA, or catalytic RNA.

23. The bacterial bleb of Claim 21, from a bacterium selected from bacteria of the group consisting of *Shigella* spp., *Salmonella* spp., *Neisseria* spp., *Haemophilus* spp., *Vibrio* spp., and *Escherichia* spp.
24. The bacterial bleb of Claim 21, wherein said bacteria are attenuated.
25. The bacterial bleb of Claim 21, from a bacterium selected from bacteria of the group consisting of *Yersinia* spp., *Escherichia* spp., *Klebsiella* spp., *Bordetella* spp., *Neisseria* spp., *Aeromonas* spp., *Francisella* spp., *Corynebacterium* spp., *Citrobacter* spp., *Chlamydia* spp., *Hemophilus* spp., *Brucella* spp., *Mycobacterium* spp., *Legionella* spp., *Rhodococcus* spp., *Pseudomonas* spp., *Helicobacter* spp., *Bacillus* spp., *Leishmania* spp. and *Erysipelothrix* spp. which have been genetically engineered to mimic the bleb-producing properties of *Shigella* spp., *Salmonella* spp., *Neisseria* spp., *Haemophilus* spp., *Vibrio* spp., and *Escherichia* spp.
26. The bacterial bleb of Claim 25, wherein said bacteria are attenuated.
27. The bacterial bleb of Claim 21, further comprising a prokaryotic expression cassette.

28. A transformed animal cell including a bacterial bleb as in claim 21, or a transfected product of said bacterial bleb.
29. The transformed animal cell of Claim 23, wherein said animal cell is a mammalian cell.
30. The transformed animal cell of Claim 23, wherein said animal cell is selected from the group consisting of humans, cattle, sheep, goat, horse, donkey, primate, and buffalo cells.
31. The transformed animal cell of Claim 23, wherein said animal cell is a human cell.
32. A method of delivering a eukaryotic expression cassette to an animal cell, comprising mucosal transfection.
33. A method of therapeutically treating an animal with a therapeutic agent encoded by a polynucleotide coding sequence, comprising transfecting the animal with a bacterial bleb containing a eukaryotic expression cassette including said polynucleotide coding sequence.
34. The method of Claim 33, wherein said polynucleotide coding sequence encodes a therapeutic agent selected from the group consisting of vaccine antigens, gene therapeutic agents, immunoregulatory agents, antisense RNA, and catalytic RNA.

35. The method of Claim 33, wherein said transfecting includes mucosal transfection.
36. The method of Claim 33, wherein said animal is a mammalian animal.
37. The method of Claim 33, wherein said animal is a human.
38. The method of Claim 33, wherein said transfecting includes administration by one or more of intravenous administration, intramuscular administration, intradermal administration, intraperitoneal administration, peroral administration, intranasal administration, intraocular administration, intrarectal administration, intravaginal administration, oral administration, immersion administration, and intraurethral administration.

Figure 1
Serum IgG responses to Salmonella after vaccination with non-pyrogenic *Salmonella* Blebs

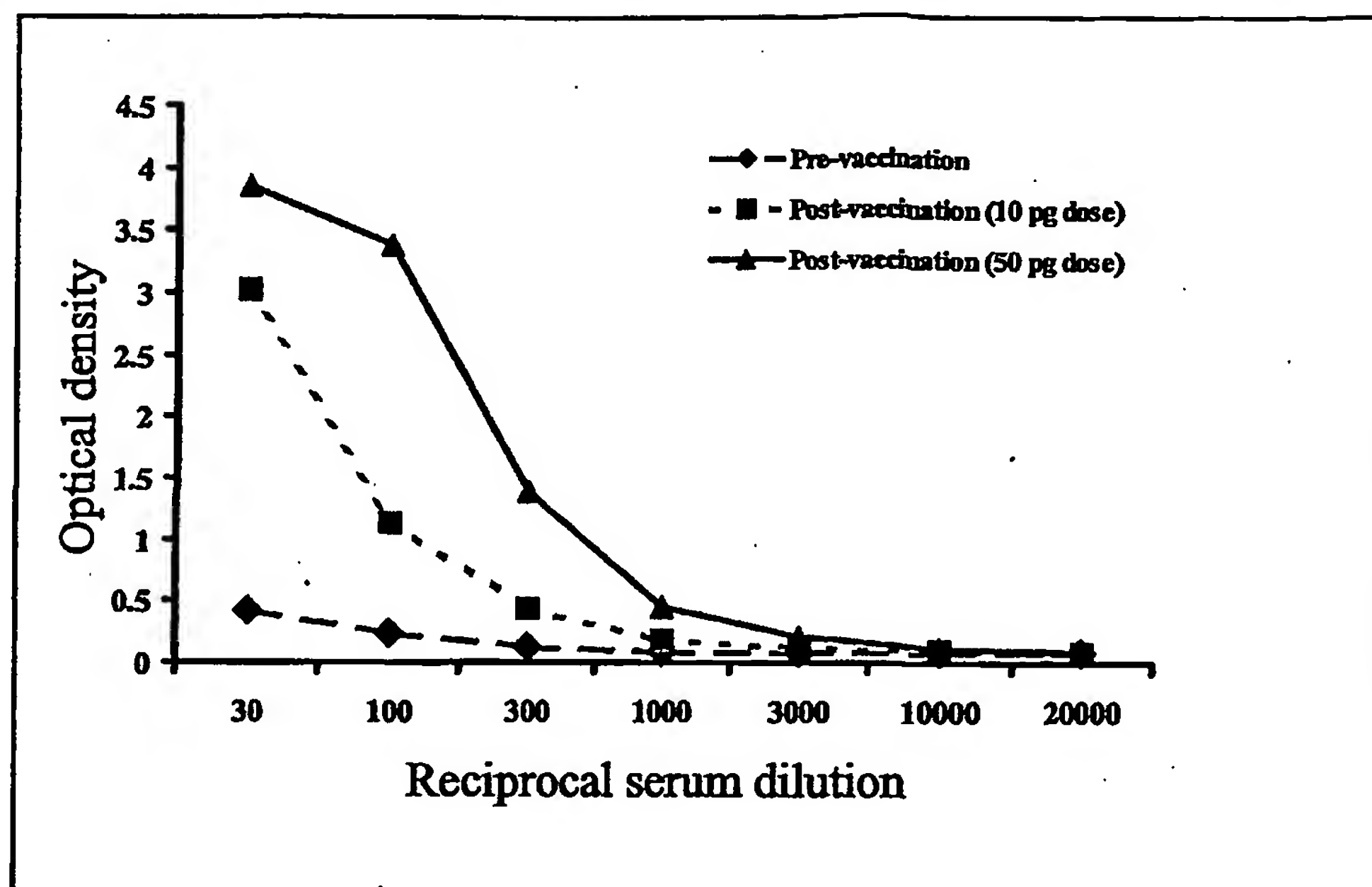


Figure 2
Serum IgG responses to gp120

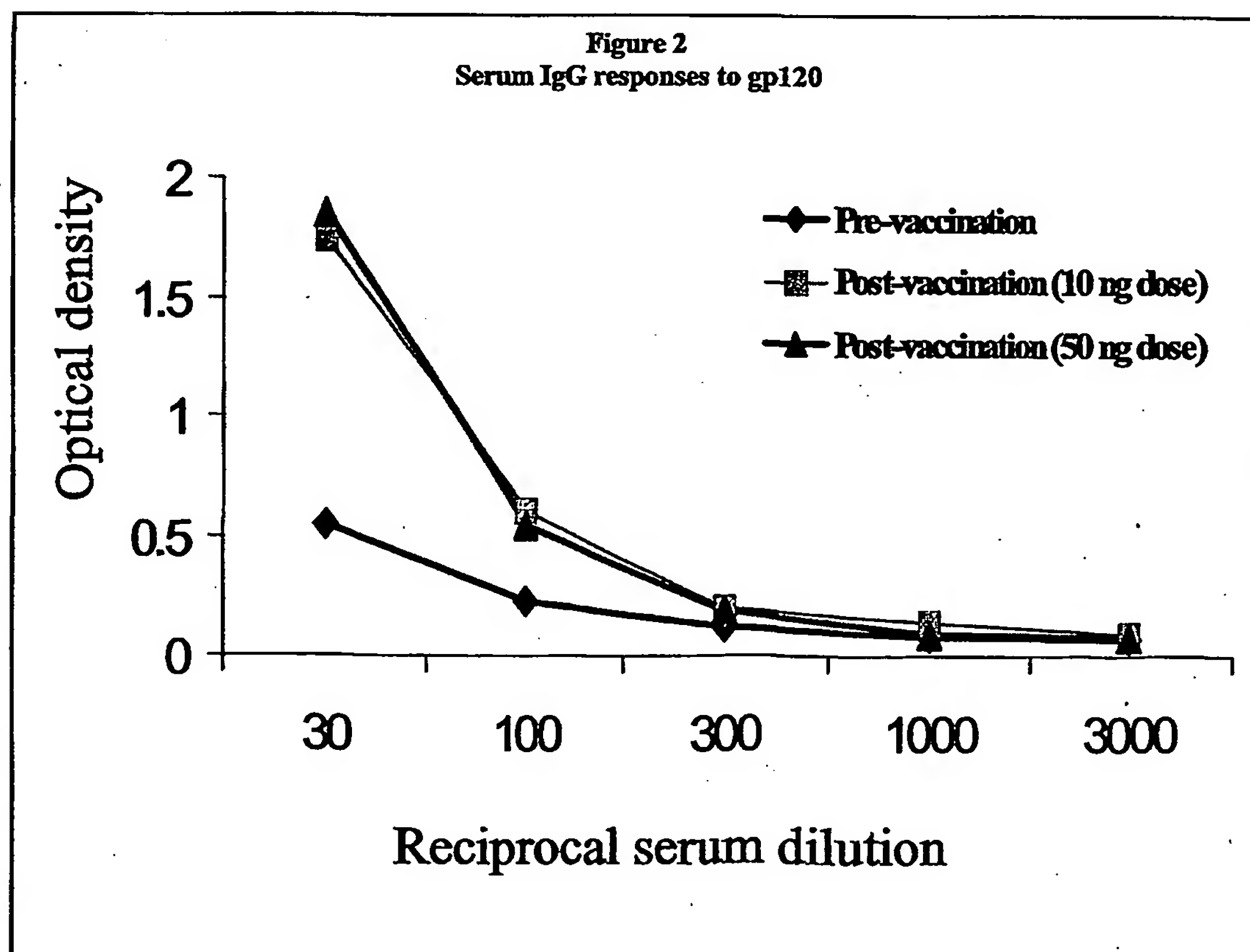


Figure 3

Cholera Toxin A1 subunit sequence

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SEQUENCE LISTING

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US01/16904

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A61K 35/00
US CL : 424, 93.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : Class 424, subclass 93.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DORWARD et al, Export and intercellular transfer of DNA via membrane blebs of	1-21
—	Neisseria gonorrhoeae. Journal of Bacteriology. May 1989, Vol 171, No.5, pages 2499-	—
Y	505, (see entire reference).	22-38
X	WO 99/59625 A1 (UNIVERSITY OF MARYLAND) 25 November 1999, see entire	1-38
—	reference.	—
X	FIOCCA et al, Release of Helicobacter pylori vacuolating cytotoxin by both a specific	1-21
—	secretion pathway and budding of outer membrane vesicles, J. of Pathology, Jan 1999,	—
Y	Vol. 188, pages 220-226, see entire reference.	22-38
X	KADURUGAMUWA et al, Delivery of the Non-Membrane-Permeative antibiotic	1-26
—	Gentamicin into mammalian cells by using Shigella Flexneri membrane vesicles.	—
Y	Antimicrobial Agents and Chemotherapy, June 1998, Vol. 42, No. 6, pages 1476-1483,	27-38
—	see entire reference.	—
X	PARKER et al, Cell surface antigens of Bordetella Pertussis, Developments in Biological	1-14
—	Standardization, 1985, Vol. 61, pages 123-126, see entire reference.	—

☒ Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"B" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T"

Later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X"

document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y"

document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&"

document member of the same patent family

Date of the actual completion of the international search

25 July 2001 (25.07.2001)

Date of mailing of the international search report

18 OCT 2001

Name and mailing address of the ISA/US

Commissioner of Patents and Trademarks

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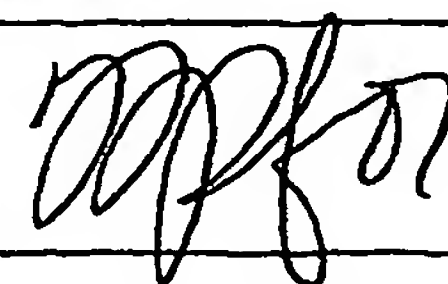
Washington, D.C. 20231

Facsimile No. (703)305-3230

Authorized officer

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US01/16904

C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BEVERIDGE et al, Periplasm, periplasmic spaces, and their relation to bacterial wall structure: Novel secretion of selected periplasmic proteins from <i>Pseudomonas aeruginosa</i> , Feb 1996, Vol. 2, No. 1, p. 1-8, see entire reference.	1-38

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US01/16904

Continuation of B. FIELDS SEARCHED Item 3:

STN: Medline, EMBase, CAPus, SciSearch, BIOSIS, Registry

search terms include: bacteria, bleb, vesicle, nucleic acid, transfection, delivery, animal, cassette, expression, eukaryotic, transformation.